

**Management strategies to control sexual maturation in  
sea-reared Atlantic salmon (*Salmo salar* L.):  
Biomass management, light-manipulation and sterility**

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## DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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## ABSTRACT

Pre-harvest sexual maturation in farmed Atlantic salmon, *Salmo salar*, remains a key biological bottleneck compromising biomass and financial output, production predictability, environmental respect, stock welfare and the overall sustainability of the on-growing industry. The management practices currently in place are not optimized and events of high maturation rate are still sporadically observed. From an ecological perspective, the escape of reproductively competent, domesticated Atlantic salmon constitutes a threat to the integrity of wild stocks. The forecasted expansion of the Scottish salmon industry compels the need for a comprehensive and more reliable control of sexual maturation. The general aim of this research project was to optimize the current management strategy (windows of light-manipulation and quality grading) and test alternative practices (lighting-technologies, selective harvest and triploidization) in the control of pre-harvest sexual maturation within the Atlantic salmon on-growing industry.

In that end, a number of trials were performed using stock reared in sea-cages on a full commercial-scale or in tanks on an experimental scale. The results of this project are organized around three experimental chapters dealing consecutively with body-size dimorphism, grading and harvest quality; light manipulations and triploidy. In each chapter, two original manuscripts either published or *in review* are included. In addition to these experimental results, a literature review chapter composed of two review papers on the photoperiodic synchronization and developmental regulation of maturation in salmonids and on morphological skin colour changes in teleosts (published) are presented.

In the first experimental chapters, we aimed at investigating the possibility of detecting and selectively harvesting a high proportion of sexually recruited fish before flesh quality deterioration. Results clearly showed that body-size dimorphisms between maturity cohorts at the end of the anabolic window of reproduction (June/July) are strong and standard predictors of maturation among related populations with the same freshwater history. Dimorphism can therefore be modelled to easily and accurately

estimate maturation rate in a number of discrete rearing-units. If required, a high proportion of sexually recruited fish can be selectively harvested as superior quality product while leaving the immature fish for further on-growing. This provides an alternative to visual grilse grading that is not feasible in large-scale aquaculture systems, prevents downgrading and increases production predictability as compared to emergency harvests. Furthermore, our results showed immature males grow faster than immature females which should be further investigated to fully determine gender specific performances and nutritional requirements. Weight-grading performed earlier in the cycle affects the sex-ratio within individual pens and in turn apparent performance. This work also revealed that Atlantic salmon can exhibit significant variations in skin colouration resembling the onset of nuptial display but that are not related to sexual recruitment and do not correlate with reduced flesh quality. This originates from a lack of purine (silver) pigments which was also identified, to a larger extent, as characteristic of the nuptial display. This suggests a degree of desmoltification in these histologically immature fish. The instrumental colouration of the altered phenotype was shown to be improved towards a more silver-like appearance by direct ice-contact. This knowledge could facilitate post-harvest quality grading towards the most appropriate market channel and increase product acceptance and attractiveness.

The second experimental chapter investigated the possibility of improving photoperiodic manipulation used to suppress early maturation, currently applied for 6-months during the second winter at sea using wide-spectrum, high-intensity lighting systems. Our results showed that the window of continuous artificial-light (LL) exposure can be reduced to 4-months following its onset in early January without compromising its efficiency in suppressing pre-harvest maturation. In addition, alternative lighting technologies were also highly potent at suppressing sexual maturation. The mean-irradiance (intensity) generated within a commercial sea-cage was inversely proportional to the suppression of nocturnal plasma melatonin (light perception hormone) and negatively correlates with the maturation rate within the commercial sea-pen. Threshold levels of light-intensity required to achieve optimal (total) suppression of sexual maturation are suggested. Alternative, narrow band-width

lighting-technologies (cold cathode and light-emitting diodes) present an array of technical, practical, economic and welfare benefits comparing to the system currently in use. Clear improvements of the photoperiod-manipulation strategy were demonstrated and these would reduce economic and environmental costs but also potential impacts on animal welfare.

The third experimental chapter showed the strong potential of sterile-triploid Atlantic salmon stocks both in freshwater and seawater. Triploid out-of-season smolts were produced for the first time using a classical accelerated "square-wave" photoperiod. Triploidization affected the smoltification pattern but had no detrimental effects on freshwater and early seawater performances under both a S0+ and S1 regime. This illustrates the need to adapt the timing of seawater transfer for successfully producing triploid Atlantic salmon post-smolts. Following one year of seawater rearing, the prevalence of external deformities was higher in triploids but remained within acceptable levels. Importantly, the incidence of vertebral deformities and ocular cataract was higher in triploids possibly due to their specific requirements. It is suggested that tailoring the diet to the nutritional requirements of triploids holds strong potential for remediation. This must be addressed if the use of sterile-triploid stock is to become a commercial reality.

The present research project provides means to optimize the maturation management strategy within the Atlantic salmon on-growing industry through light-manipulation, maturation detection and selective harvest, and quality grading. Proposed improvements have the potential to increase biomass and financial output, production predictability, environmental respect and animal welfare and will allow standardization of the overall control of pre-harvest sexual maturation. Their implementation provides a comprehensive strategy likely to favour a sustainable expansion of the Atlantic salmon industry. From a longer term perspective, the rearing of sterile-triploid stocks is promising and should be actively investigated to isolate domesticated strains from their wild conspecifics. This would also eliminate the need for on-growers to deploy a maturation management strategy that that might still affect stock welfare and remains, despite the strong improvements demonstrated, not 100% reliable, costly, technical and protracted.

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## LIST OF ABBREVIATIONS

$\alpha$ -MSH	Alpha-melanocyte-stimulating hormone
$\alpha$ MSHR	Alpha-MSH receptor
a*	Red-green chromacity
AANAT2	Arylalkylamine N-acetyltransferase
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AMP	Adenosine Monophosphate
ARA	Arachidonic acid
ASA	(gill) Arch surface area
ATP	Adenosine triphosphate
Ax	Astaxanthin
B	50W 'blue' light-emitting-diode
b*	Yellow-blue chromacity
BCA	Bicinchoninic acid
BH <sub>2</sub>	Dihydrobiopterin
BH <sub>4</sub>	Tetrahydrobiopterin
BPG	Brain-pituitary-gonadal
BSA	Bovine serum albumin
BW	Whole body-weight
C	Cortisol (Paper II)
C	400 W 'white' metal-halide (Paper VI)
CA	Cortical alveoli stage
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CSI	Cardio-somatic index
Cx	Canthaxanthin
D	Dermis (Paper I) or Dorsal (Paper IV)
Dio2	Deiodinase
DV	Dilution volume
E	Erythrophore (Paper I; See figure legends)
E <sub>2</sub>	Oestradiol-17 $\beta$
F	Family
FL	Fork length
FW	Freshwater
IMP	Inosine Monophosphate
G	Guanine or 232 W 'green' hot cathode (Paper VI)
GFD	Gill filament deformity syndrome
GFDS	Gill filament deformity syndrome
GH	Growth hormone
GPR54	Protein receptor 54
GMP	Guanosine Monophosphate
GnRH	Gonadotrophin-releasing hormone
GnRH <sub>a</sub>	Gonadotrophin-releasing hormone analog
GSA	Gill surface area
GSI	Gonado-somatic-index
GTP	Guanosine Triphosphate
GTPCH	GTP cyclohydrolase I
GW	Gonad weight
H	Hypoxanthine.
HPA	Hypothalamic-pituitary-adrenal
HPI	Hypothalamic-pituitary-interrenal
HPLC	High performance liquid chromatography

HW	Heart weight
Hx	Hypoxanthine
I	Iridophore
I*	Crystalline platelet
IGF	Insulin-like growth factor
IIY	Tertiary yolk stage
IY	Secondary yolk stage
IMP	Inosine monophosphate
inhib	Inhibition
InterVS	Intervertebral space
IXP	Isoxanthopterin
IY	Primary yolk stage
K	Fulton's condition factor
L	Leucophore
L*	Colour lightness
LDH	Lactate dehydrogenase
LED	Light-emitting-diode
LJD	Lower jaw deformity
LJI	Lower jaw indew
LD	Light-Dark
LH	Luteinizing Hormone
LL	Continuous (artificial) light
M	Melanophore
MCH	Melano-concentrating-hormone
MC1R	Melanocortin 1 receptor
MC2R	Melanocortin 2 receptor
MERL	Machrihanish Marine Environmental Research Laboratory
m	Melanophore or melanoblast (Paper I; See figure legends)
M	Muscle or Melanophore (Paper I; See figure legends)
M*	Melanosome
mRNAs	Messenger ribonucleic acids
MSHc	$\alpha$ MSH producing cell
n	Nucleus
NADH/NAD+	Nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide
NAS	N-acetyl-serotonin
NBFRF	Niall Bromage Freshwater Research Facility
NE	Norepinephrine
NIR	Near-infrared reflectance spectroscopy
NL	Natural photoperiod
NO	Nitric oxyde
NPY	Neuropeptide Y
NQC	Norwegian quality cut
OD	Oil drop stage.
p.-oxyd	Photo-oxidation
Pg	Progesterone
PEP	Phosphoenolpyruvate
PhenylH	Phenylalanine hydroxylase
PK	Pyruvate kinase
PNES	Photo-neuro-endocrine system
POMC	Pro-opiomelanocortin
prec	Precursor
P	Purine crystal
PRL	Prolactin
PRLR	PRL-receptors

PT	Pars tuberalis
PTPS	6-pyruvoyl tetrahydropterin synthase
PUFAs	Polyunsaturated fatty acids
R	(vertebral) Region (Paper VIII) or 400W 'red' tungsten-halogen (Paper IV)
RBC	Red blood cell
Rep	Replicate
SQC	Scottish quality cut
SW	Seawater
SWT	Seawater transfer
RMA	Reduced major axis
RWG	Round weight gain
s.a.	Specific activity
SA	Stratum argenteum
SAD	Seasonal Affective Disorders
Sc	Scale
SC	Stratum compactum
SE or SEM	standard error standard error of the mean
SGR	Specific growth rate
SL	Somatolactin
SLc	SL producing cell
SMIPs	Suprachiasmatic-melanotrope-inhibiting neurons
SNP	Simulated natural photoperiod
SPR	Sepiapterine-reductase
SS	Stratum spongiosum
SSD	Sexual size dimorphism
S0+	Out-of-season smolt
S1	In-season smolt
T	Testosterone
TGC	Thermal growth coefficient
TH	Thyroid hormone
trypH	Tryptophan hydroxylase
TSH	Thyroid-stimulating hormone
TyrH	Tyrosine hydroxylase
Tyr	Tyrosinase
T <sub>3</sub>	Triiodothyronine
T <sub>4</sub>	Tetraiodothyronine or thyroxine
V	Vertebrae (Paper VII) or Ventral (Paper IV)
WLR	Weight length relationship
x or X	Xanthophore
XDH	Xanthine dehydrogenase
XMP	Xanthosine Monophosphate
XO	Xanthine oxydase
XP	Xanthopterin
1-or 2-SW	One or two seawinter
11-KT	11-ketotestosterone
2N	Diploid
3N	Triploid
<sup>3</sup> H-T	Tritiated-testosterone

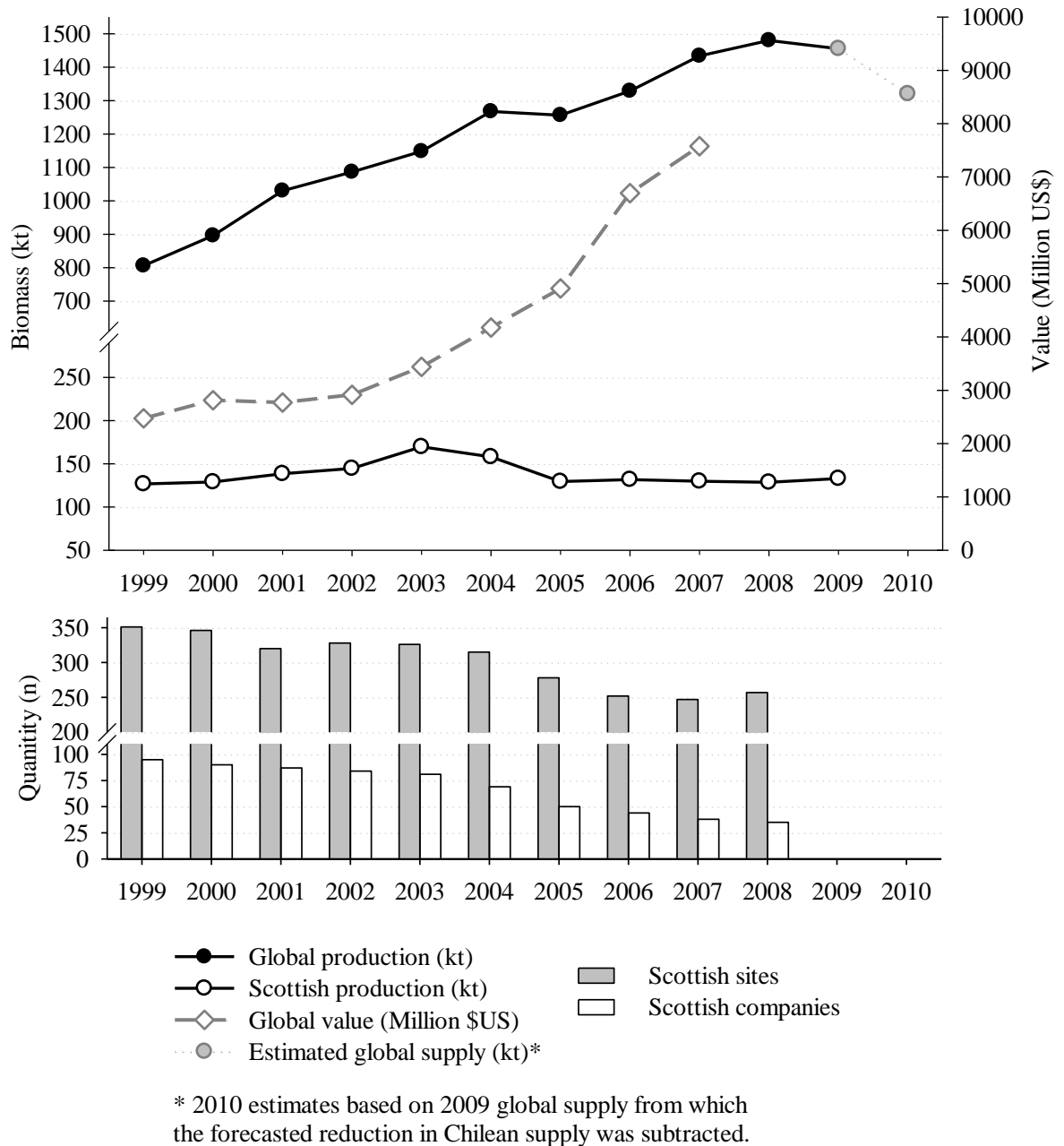
**CHAPTER 1**  
**GENERAL INTRODUCTION**

## CHAPTER 1 GENERAL INTRODUCTION

### 1. The Atlantic salmon industry

Within the last 8 years, the global aquaculture production of Atlantic salmon, *Salmo salar*, has increased 1.8 fold in volume (+78% from 806 kt to 1434 kt) and 3-fold in value (+ 206%, from 2.5 to 7.6 billion US\$) confirming Atlantic salmon as the world leading finfish aquacultural species in terms of value (FAO, 2009). In Scotland, production volume increased 4.4-fold from 1989 to 1999 (+344% from 29 kt to 127 kt respectively) but only 1.05-fold from 1999 to 2009 (+5% from 127 kt to 133 kt; Marine Scotland, 2009; Fig.1a). Despite a relatively stable production volume over the last decade and motivated by strong annual variations in salmon price (data not shown), the Scottish salmon industry has pursued its consolidation toward an ever increasing scale of operations. This is shown by the continuing trend toward fewer but larger sites and companies in both fresh- and sea-water. Between 2000 and 2008, the number of companies involved in Scottish Atlantic salmon on-growing decreased by 67% to 35 and the number of sea-sites by 25% to 257 (Fig.1b). As a result, 95% of the Scottish Atlantic salmon harvested in 2008 originated from 9 companies producing more than 2000 t a year and 76% from 57 sea-sites producing over 1000 t a year (Marine Scotland, 2009). With the global demand for Atlantic salmon steadily increasing (FAO, 2009) and, due to the recent collapse of the Chilean salmon industry (650 kt harvested in 2008, 400 kt in 2009 and forecasted 100 kt in 2010), there is an estimated global shortage of 190 kt of Atlantic salmon in 2010 (Intrafish, 2010a and b). This situation favours high market price, thereby increasing the value of the Scottish production, and this is forecasted to prevail for the next five years until the Chilean industry output returns to its historical level (Intrafish, 2010c). Following a decade of industrial consolidation and

stable production volume, the Scottish government and the Scottish Salmon Producers Organization (SSPO) are now planning to increase the national output by 23% in the next five years, from 133 kt to 160 kt (Scottish Government, 2010; Intrafish, 2010c).



**Figure 1.** (a.) Global and Scottish annual production of Atlantic salmon. (b.) Number of sites and companies involved in Atlantic salmon on-growing in Scottish sea-waters.



The consolidation of the Scottish salmon industry favours the implementation of standard rearing practices and presents, for any companies, the opportunity of higher gains as well as the risk of heavy losses from variations in key zootechnical performances. In order to achieve long-term growth under potentially deteriorating market conditions, the industry must optimize stock performance at all levels and fully benefit from its scale. Importantly, it must continually improve sustainability by ensuring full stewardship toward environmental and welfare issues. Pre-harvest sexual maturation remains a key biological bottleneck compromising biomass and financial output, production predictability, environmental respect, animal welfare and the overall sustainability of the industry.

## **2. Dynamic of oogenesis in Atlantic salmon**

In female teleosts, four main stages of oogenesis are typically distinguished: the primary growth, cortical alveoli or endogeneous yolk formation, vitellogenesis (exogeneous yolk formation and accumulation in the oocyte) and final maturation (Wallace and Selman, 1981; Tyler and Sumpter, 1996) which is followed by ovulation and spawning. Three oocyte growth phases are also typically distinguished. The primary growth phase that includes all pre-vitellogenic oocyte stages, the secondary growth phase that relates to endogenous and exogenous vitellogenesis and the tertiary growth phase corresponding to final oocyte maturation and hydration. Stages of oocyte development are presented in Table 1. (Bromage and Cumaranatunga, 1988, Taranger, 1999).

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**Table 1.** Stages of oogenesis in Atlantic salmon (Bromage and Cumaranatunga, 1988, Taranger, 1999).

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- ***The primary growth-phase: Pre-vitellogenic oocyte growth***

***I Chromatin nuclear stage***

The central nucleus increases in diameter, chromatin strands appear within the oocyte and nucleoli initiate multiplication.

***II, III Early and late perinucleolar stage***

Nucleoli multiply around the periphery of the nucleus, the cytoplasm becomes progressively less basophile and the follicular layer initiates development.

---

- ***The secondary growth-phase: Vitellogenic growth***

***IV Cortical alveoli or vesicle stage followed by the Oil drop stage***

Endogeneous yolk vesicles appear in the oocyte periphery. This is followed, at the oil drop stage, by an accumulation of endogeneous yolk droplets in the perinuclear area. The follicular layer differentiates into the theca and the granulosa; the zona radiata (constituting the future egg shell) initiates development between the granulosa and the highly acidophilic ooplasm.

***V Primary yolk stage: Initiation of true vitellogenesis***

Small globules of exogenous (true) yolk are accumulated at the periphery of the ooplasm.

***Secondary yolk stage***

Yolk globules are increasingly accumulated throughout the oocyte. The follicular layers are well developed and the oocyte nucleus remains central.

***VI Tertiary yolk stage: Completion of true vitellogenesis.***

Yolk globules are poorly distinguishable as they fill the whole oocyte. The germinal vesicle (GV) initiates its migration towards the oocyte periphery, the zona radiata constitutes a bilayer with a distinct zona interna and zona externa.

---

- ***The tertiary growth-phase: Final oocyte maturation***

***VII Germinal vesicle migration and break-down***

The germinal vesicle (GV) completes its migration toward the follicular wall. The GV nuclear membrane breakdown (GVBD) occurs, the yolk mass liquefy and clarify. The oocyte undergoes hydration inducing a characteristic rapid oocyte growth. In salmonids, mature oocyte are then expelled into the abdominal cavity, a process known as ovulation.

***VIII Post-ovulatory follicles and atretic***

The hypertrophied follicular layers collapse in the follicular lumen. Non-expulsed mature oocytes degenerate as “post-ovulatory atretic oocyte”.

---

Inherent to their temperate and sub-polar geographical distribution (Murua and Saborido-Rey, 2003), Atlantic salmon are “total spawners” or “synchronous ovulators” shedding the whole annual batch of oocytes in a close series of 2 or 3 events within a relatively short spawning window of 1 to 2 weeks in late autumn/early winter (Tyler and Sumpter, 1996). Unlike Pacific salmon, Atlantic salmon are iteroparous or successive spawners reproducing more than once in their life-time. Accordingly, they have a group-synchronous ovarian type where two to three main groups of oocytes occur simultaneously in developing ovaries (Wallace and Selman, 1981). The less developed oocyte group is made of oogonia undergoing primary oocyte growth and the second of oocytes undergoing endogenous vitellogenesis. This last group constitutes a “reserve fund” of pre-vitellogenic oocytes from which a leading oocyte “clutch” is recruited, undergoes exogenous vitellogenesis in close synchrony and constitutes the current-season batch of eggs (Bromage and Cumaranatunga, 1988; Bromage *et al.*, 1992). Salmonids have a cystovarian (closed) ovary structure characterized by a true ovarian capsule where oocytes are ovulated into the intraovarian space or oviduct (Dodd, 1977).

The initiation of exogenous vitellogenesis is of particular interest within the annual reproductive cycle of Atlantic salmon. In a population of first-time spawners (all females at the cortical alveoli or oil drop stage in late February), gonadal development was interrupted at the oil drop stage in fish remaining subsequently immature while females that completed maturation in the on-going season initiated exogenous vitellogenesis in late April (Taranger *et al.*, 1998, 1999). The onset of true vitellogenesis (Primary yolk stage) can therefore be used as an indicator of recruitment into maturation in female Atlantic salmon while, in the male population, previous studies typically

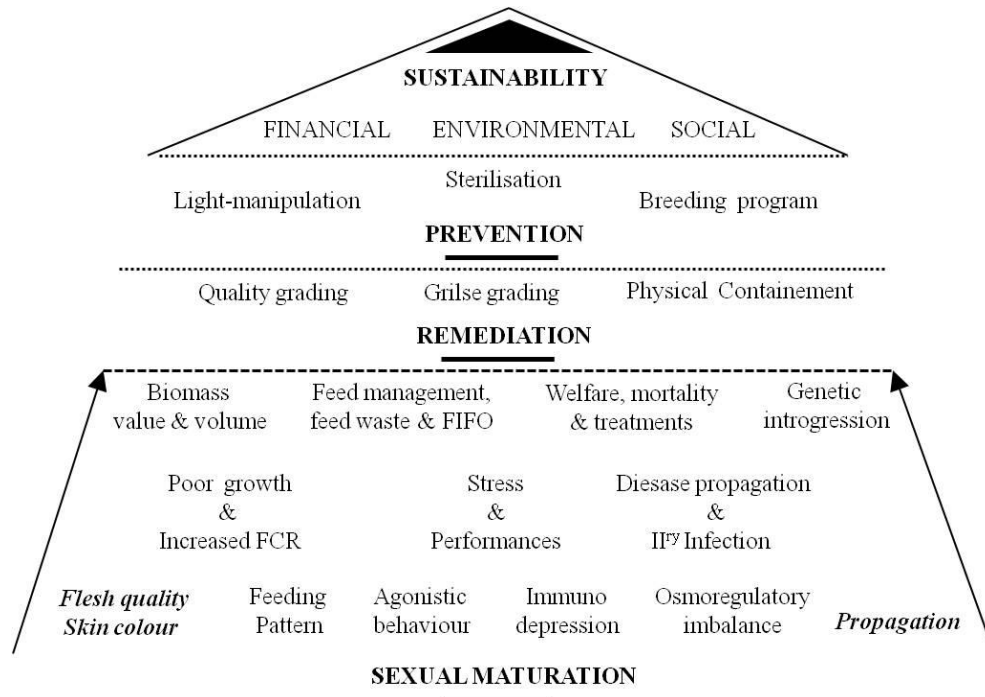
used the bimodal gonado-somatic-index (GSI) distribution to characterize sexual maturity (Kadri *et al.*, 1997a and b; Taranger *et al.*, 1998; Endal *et al.*, 2000). Based on GSI, the onset of male maturation was reported in June (compared to late May in female) followed by rapid gonadal growth particularly in males that completed 90% of their maximum GSI in August compared to October in females (Aksnes *et al.*, 1986).

In terms of sex-steroid levels (Hunt *et al.*, 1982; Taranger *et al.*, 1998, 1999), plasma-testosterone (T) and oestradiol-17 $\beta$  (E<sub>2</sub>) remain basal at levels below 1 ng.ml<sup>-1</sup> from February to July in immature fish. In the maturing cohort, plasma-T significantly increases from February onward reaching levels of circa 3 ng.ml<sup>-1</sup> in May-June and above 5 ng.ml<sup>-1</sup> from July onward. Similarly, plasma-E<sub>2</sub> is significantly higher and increases progressively in maturing fish from February to July.

### **3. The negative impacts of sexual maturation**

The rapid somatic growth experienced by Atlantic salmon in seawater is a key feature of the species life-strategy ultimately serving to fuel and maximize reproductive success and gonadal growth in particular. Sexual maturation, which can be defined as the transition to a developmental status capable of reproduction, is a broad developmental process encompassing changes at all levels of the organism and particularly so in anadromous salmonids migrating back into freshwater to spawn. Within the on-growing industry, pre-harvest sexual maturation is accordingly the prime endogenous event compromising the performance of the stock through a range of negative effects (Fig.2).

The onset of sexual development, characterized by an increase in plasma sex steroid levels with little gonadal growth is concomitant with an increased appetite and growth rate from January to June (Taranger *et al.*, 2010). This defines the anabolic



**Figure 2.** Schematic representation of the range of negative effects of sexual maturation together with the remediation and preventive measures available to achieve sustainability.

window of maturation leading to a peak in body-size dimorphism between maturity cohorts in early summer (Kadri *et al.*, 1996, 1997a and b). Under domestication, the inherent monopolization of food by the maturing fish at an early developmental stage is likely to compromise the feeding opportunities and thereby the growth-rate and condition factor of the cohabiting immature cohort (Kadri *et al.*, 1997a). Later on from early summer onward, maturing fish exhibit a rapid drop in appetite concurrent with a reduced growth or even a decrease in body-weight (Kadri *et al.*, 1997b). This anorexic status is concomitant with a mobilization and a redistribution of the somatic resources to support the rapid gonadal growth experienced at this stage (Taranger *et al.*, 2010). It was indeed shown at this stage that the maturing ovary had the highest amino-acid requirements and that these were derived from a degradation of the white muscle proteins (Martin *et al.*, 1993). These events define the catabolic window of sexual maturation. For the on-grower, the transition from a catabolic (high feeding rate) to an

anabolic (low feeding rate) window in a proportion of the stock runs the risk of overfeeding, feed waste, poor food conversion ratio and increased organic pollution.

More directly, the poor flesh quality and skin colour exhibited by sexually mature fish over the catabolic window of maturation (from mid-July/August onward) is responsible for their downgrading during post-harvest quality grading (Michie, 2001). Downgraded fish are lost for human consumption and constitute a direct loss to the industry. The pattern of deterioration in different flesh quality parameters was previously described (Torrissen and Torrissen, 1985; Aksnes *et al.*, 1986; Martin *et al.*, 1993; Nickel and Springate, 2001). In particular, the onset of nuptial metamorphosis (deterioration of skin colouration) was reported in late July followed by a significant reduction in flesh redness, protein and fat content from September onward (Aksnes *et al.*, 1986). Sexual maturation is also concomitant with the expression of agonistic behaviour, a likely osmoregulatory imbalance under containment and immune-depression (Traxler *et al.*, 1997; St-Hilaire *et al.*, 1998; Currie and Woo, 2007). The mature cohort is likely to be increasingly sensitive to occurring pathogens and may act as a disease vector and reservoir toward the immature cohort although this remains poorly quantified to this day. Holding fish over the catabolic window of sexual maturation is likely to significantly increase the risk of disease outbreak and the need for chemotherapeutic treatments, particularly considering that immature fish might be physically (attack) or physiologically (stress) compromised as a result of the agonistic behaviour of the mature cohort.

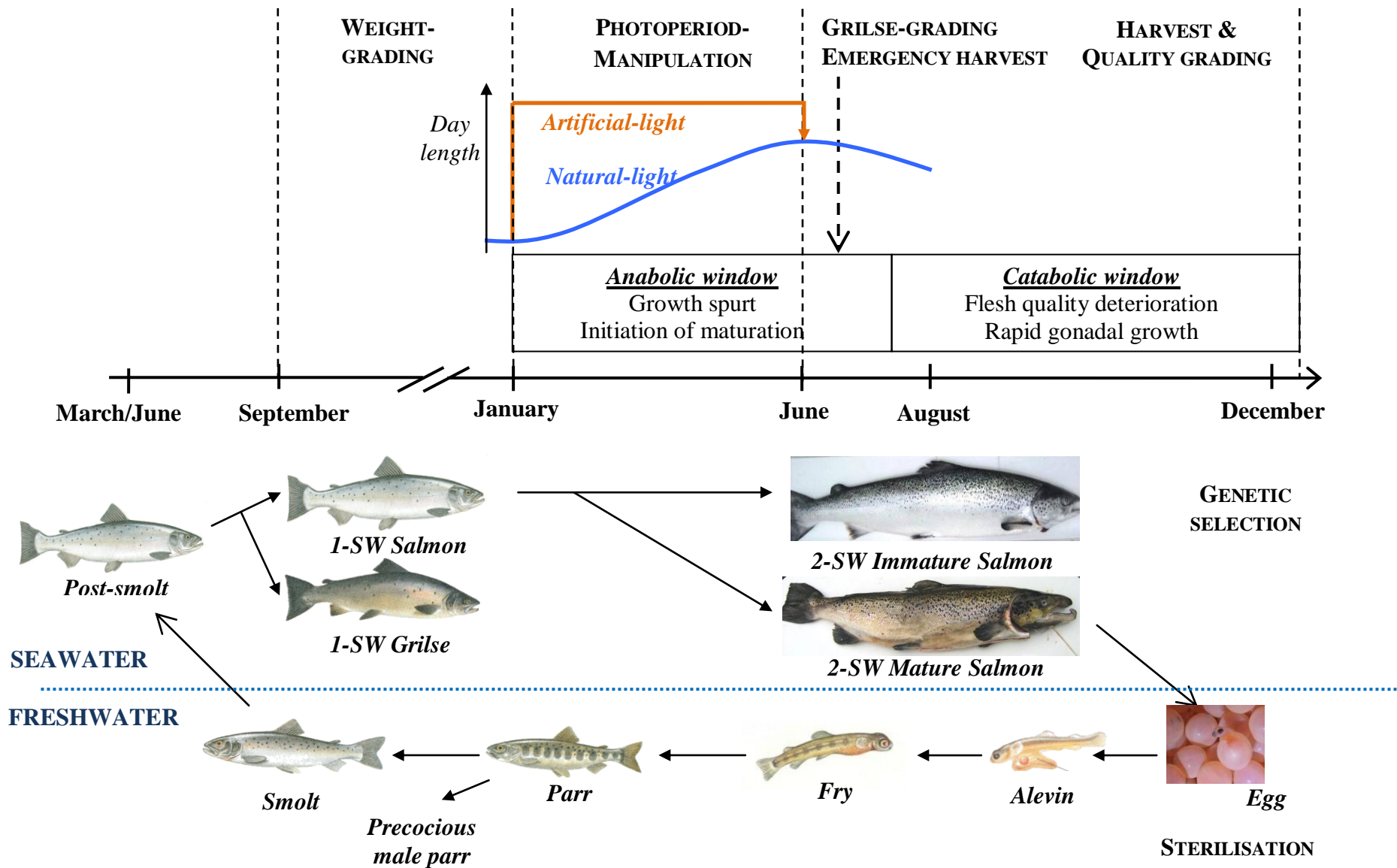
Finally, domesticated strains of Atlantic salmon typically show a reduced genetic variation (Skaala *et al.*, 2005) but are capable of natural reproduction upon escape (Webb *et al.*, 1991; 1993). Within the species native range, escapes could reduce the fitness of wild populations through wild-farmed fish hybridization (Crozier 1993;

McGinnity *et al.*, 1993, 2003; Skaala *et al.*, 2006; Roberge *et al.*, 2008). When reared outside their native range, the propagation of self-sustaining strains of non-native Atlantic salmon could significantly interact with the indigenous biota in a usually irreversible manner (Beveridge, 1996; Arthington and Blühdorn, 1997).

Sexual maturation has a wide range of direct and indirect negative effects compromising the profitability, predictability, environmental footprint, animal welfare and sustainability of the industry. In eastern Canada, pre-harvest sexual maturation was said to be increasing in the past decades to an estimated 12% average prevalence rate and to constitute one of the largest economic problems for the salmon industry in this area (McClure *et al.*, 2007). In Scotland, maturation of Atlantic salmon was reported to account for, on average, 5% of the harvested stock and to be the single most significant cause of downgrading in 1998 (Michie, 2001). This would be equivalent to a downgraded biomass of circa 3250 t when adjusted to 2007 Scottish production volume. Although pre-harvest maturation rates across the industry are likely to have been reduced nowadays, localized events of high maturation (>10%) are still sporadically observed. When such events occur, the range of negative impacts are likely significant with devastating consequences for the site affected.

#### **4. Management of sexual maturation in the Atlantic salmon on-growing industry**

The suppression of sexual maturation is therefore a priority in the industry and this is achieved through a range of management practices applied at different stages of the rearing cycle. Each practice presents its own advantages, disadvantages and efficiency such that they remain, to this day, often complementary to one another. They are schematically presented in relation to the production cycle of Atlantic salmon in Fig.3.



**Figure 3.** Schematic representation of the production-cycle of Atlantic salmon stocked at sea in-season or slightly out-of-season where the problem of pre-harvest maturation is most significant. The main practices available to reduce sexual maturation and its negative effects are highlighted with an indicative time-line.



#### **4.1. Genetic selection**

Selective breeding programs are established across all major producing countries but related data are typically poorly available. The first Norwegian salmon breeding program was initiated in 1975 with selection for growth to which age at sexual maturation was included in 1981. In comparison, selection for disease resistance, flesh colour and carcass composition was incorporated from 1993 (Gjøen and Bentsen, 1997). In this report, the genetic gain per generation (4 years) for age at maturation was estimated at 10% with a heritability of  $0.48 \pm 0.20$  in a previous study (Gjerde, 1984). However, data communicated by AquaGen<sup>TM</sup> (2005) show that sexual maturation was given a partial weighting of only 5% when determining the breeding value of candidate 3 sea-winter broodstock in 2001 (AquaGen, 2005). Selective breeding has undoubtedly contributed to reducing pre-harvest sexual maturation in farmed stock. However, there is a significant environmental component in the age at first maturation (Gjerde, 1984) and the industry still relies on a number of strains likely to show variability in their age and size at first maturation. In any case, and from an ecological perspective, such fish are fully capable of reproduction.

#### **4.2. Weight-grading and top-crop harvesting**

It is recognized that some thresholds of body-size must be surpassed for sexual maturation to proceed (Thorpe, 1994; Thorpe *et al.*, 1998) such that Atlantic salmon recruited into maturation will typically be among the best performers within a given population. As expressed in the RSPCA welfare standards for farmed Atlantic salmon, “Size grading early gives the opportunity to select potential grilse in the first harvests prior to maturation” (RSPCA, 2010). It is nowadays common practice to undertake weight-grading during the first winter at sea such that the best performers are

segregated, reared under natural photoperiod (NP) and harvested prior to the onset of the catabolic window of sexual maturation (July). This practice contributes to the low maturation rate typically observed at harvest in recent years. However, the body-size thresholds are not characterized and are likely to vary with strains, rearing conditions and timing of size-grading. Weight-grading remains empirical in that aim and is primarily performed to manage size-variability and population density. Additionally, it is not systematically undertaken but sometimes replaced by population thinning.

### **4.3. Photoperiod-manipulation**

Photoperiod-manipulation is the only management practice available to the on-grower to suppress pre-harvest maturation before its actual onset. This is typically performed in the industry by applying continuous artificial-light (LL) from the winter to the summer solstice during the second year at sea on stock to be harvested during the subsequent half of the year. The onset of LL in January has indeed been shown to be the most effective method at suppressing gonadal development by providing a key environmental signal that phase-advances the so-called 'spring decision window' such that a reduced proportion of the stock meets the developmental/energetic thresholds required to proceed through maturation (Hansen *et al.*, 1992; Thorpe, 1994; Thorpe *et al.*, 1998; Taranger *et al.*, 1998, 1999; Endal *et al.*, 2000; Bromage *et al.*, 2001; Oppedal *et al.*, 2006). Accordingly, the photoperiodic regime postpones recruitment into sexual maturation to the following year such that responding fish remain immature over the remainder of the production cycle. Becoming increasingly standardized and providing good results within the Scottish industry, photoperiod manipulation is arguably highly beneficial at all levels (financial, environmental and welfare) in comparison to the negative impacts of maturation in stock reared under NP. However, applying LL for a

6-month period using powerful wide-spectrum lighting-systems as presently done has a high-running cost but also environmental and potential welfare impacts (Migaud *et al.*, 2007a). Furthermore, the potency of photoperiodic treatments is affected by other drivers of sexual maturation (e.g. photoperiodic history, nutritional status) and by a range of environmental and husbandry parameters (e.g. water clarity; lighting-strategy and disruption). In this respect, the maturation rates experienced under commercial conditions may vary and remain difficult to predict until the onset of nuptial skin colouration.

#### **4.4. Grilse-grading and emergency-harvest**

The onset of nuptial display was traditionally used as an indicator of sexual maturation before the onset of the detrimental effects of maturation on flesh quality (at the end of the anabolic window in late June/July) in order to harvest maturing fish as superior quality biomass. “Grilse grading” consisted of subjectively assessing the external colouration of individual fish and manually segregating “grilse” from the remainder of the stock. This method was not fully reliable even when performed by experienced workers (Michie, 2001) and also stressful to the nearly fully-grown fish manipulated under high water temperature. Additionally, it was necessarily performed at the end of the anabolic window and therefore did not prevent its negative effect on the cohabiting immature cohort (feed monopolization; Kadri *et al.*, 1996, 1997a). Due to the present scale of the industry and to the overall reduced maturation rates typically observed, subjective “grilse grading” is not presently performed in most Scottish sea-sites.

However, this is often replaced by an “emergency harvest” of the pen where high maturation rates occur to allow harvest of maturing fish before they suffer a

deterioration in flesh quality. Emergency harvests can be prone to a high rate of downgrading at primary processing due to the advanced nuptial metamorphosis in a proportion of the stock. Additionally, the complete and early harvest of affected pens interferes with the production schedule and the capacity to deliver the targeted average weight since the stock is typically undersized. Finally, emergency harvest might not be an option with respect to veterinary medicine withdrawal periods and requires the immediate availability of specific resources for implementation (e.g. wellboat) which can prove problematic.

#### **4.5. Post-harvest quality grading**

Quality grading at primary processing, aiming at directing harvested fish into market channels of distinct value and quality requirements, does not prevent, as such, any of the detrimental effects of sexual maturation. It is however a critical practice to achieve customer satisfaction and optimal financial return. Performed on gutted fish, any deterioration of flesh quality due to sexual development is indirectly estimated by a subjective assessment of the nuptial display intensity. The external colouration of fish is a complex trait that can be altered by various nutritional, environmental (e.g. surrounding light conditions and water clarity; Fujii *et al.*, 2000), social (e.g. subordination and stress; O'Connor *et al.* 1999, 2000; Höglund *et al.*, 2000) and endogeneous (e.g. sexual maturation, Storebakken *et al.* 1987; Torrissen *et al.*, 1989; Bjerkeng *et al.* 1992, 2000) factors. It might also vary at a post-harvest stage (e.g. ice storage; Doolan *et al.*, 2008; Erikson and Misimi, 2008). No factors other than sexual maturation have been so far described as repeatedly affecting the skin colouration of Atlantic salmon. Sexual maturation and poor flesh quality are therefore typically

presumed when deviations from the desirable silver phenotype occur and this might lead to unnecessary downgrading reducing the value of the harvested stock.

#### **4.6. Sterilization**

The production of sterile fish by triploidization of recently fertilized eggs is the only commercially available technique to achieve complete and permanent suppression of sexual maturation. By providing genetic and reproductive containment, sterilization is a reliable means to prevent the genetic pollution of wild stocks and the propagation of non-native Atlantic salmon populations (Fleming *et al.*, 1996; Piferrer *et al.*, 2009). For the on-grower, it would have the added benefit of eliminating the negative impacts of maturation on the value, growth, health and welfare of the stock (Salte *et al.*, 1995; St-Hilaire *et al.*, 1998; Kadri *et al.*, 1997a and b; Michie, 2001; Taranger *et al.*, 2010). The rearing of sterile stocks would therefore ease biomass management by removing the need for light-manipulation and grading/harvest strategies which can be costly and hold environmental and welfare impacts themselves (Migaud *et al.*, 2007).

The main reason for the lack of commercial implementation of triploidy in the Atlantic salmon industry is the reduced performance of triploid stocks observed in previous studies and commercial testing over the last two decades (Benfey, 2001; Oppedal *et al.*, 2003). However, a strong parental effect on triploid performance was reported in various studies (Friars *et al.*, 2001; Oppedal *et al.*, 2003; Johnson *et al.*, 2004) and it is now accepted that triploid fish should be treated as a “new species” (Benfey, 2001) due to their specific environmental, nutritional and physiological needs. Triploidization is increasingly regarded as a necessary step to isolate domesticated Atlantic salmon stocks. Determining the performance of triploid stock produced from an established (diploid) selective breeding program and reared in isolation from diploids

is a preliminary requirement to assess the feasibility of triploid salmon and define more specific research programs.

## 5. Experimental aims

The current management of pre-harvest sexual maturation in the Atlantic salmon on-growing industry is not optimized and events of high maturation rate are sporadically observed. The size-grading/top-crop harvest strategy is mainly driven by imperatives of size-variability and density management. Photoperiod-manipulation is not systematically biologically effective, has a high running-cost and potential welfare impacts. Following light-manipulation, no practical management tools exist to easily estimate maturation rate which can lead to late emergency harvest. The relationship between skin colouration and flesh quality remains poorly characterized impeding post-harvest quality grading. Finally, sterilization by triploidization could constitute a unique and reliable solution to the problem of sexual maturation during on-growing but only little is known so far on the physiology of triploid fish. The forecasted expansion of the Scottish salmon industry compels the need for a comprehensive and reliable control of sexual maturation that maximises the profitability, environmental respect, animal welfare and overall sustainability of the industry.

The general aim of the thesis was to investigate and optimize the management strategies currently in place to control sexual maturation in the Atlantic salmon on-growing (short-term) and assess the commercial feasibility to on-grow sterile-triploid stocks (longer-term).

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The specific objectives of the present thesis were:

1. To provide a conceptual framework on the photoperiodic entrainment and somatic regulation of the annual reproductive cycle in salmonids (**Chapter 2, Paper I**).
2. To review the ecological origins and physiological regulation of morphological colour changes in teleosts with emphasis on salmonids (**Chapter 2, Paper II**).
3. To provide an alternative to visual “grilse-grading” and emergency harvest by investigating the possibility to estimate maturation rate and segregate maturing fish using body-size dimorphisms (**Chapter 4, Paper III**).
4. To facilitate post-harvest quality grading by improving our understanding on the relationship between skin colour, flesh quality and sexual development in Atlantic salmon (**Chapter 4, Paper IV**).
5. To test the potency of reduced windows of artificial continuous-light at inhibiting pre-harvest sexual maturation (**Chapter 5, Paper V**).
6. To test the potency of novel alternative lighting-technologies at inhibiting pre-harvest sexual maturation (**Chapter 5, Paper VI**).
7. To assess the parr-smolt transformation of triploid Atlantic salmon with emphasis on the production of out-of-season triploid post-smolts (**Chapter 6, Paper VII**).
8. To determine the seawater performances of sterile-triploid stocks, identify the key bottlenecks and define future research directions aiming at adapting husbandry practices to triploid stocks (**Chapter 6, Paper VIII**).

The present thesis is presented in the form of one review and three experimental chapters. Each chapter compiles two sections addressing a single objective in a paper format. The manuscripts have either been published (Paper II, III, IV, V), accepted (Paper VIII), submitted (Paper VI, VII) or are ready to be submitted (Paper I) in peer reviewed journals.

**CHAPTER 2**  
**LITTERATURE REVIEW**



## CHAPTER 2

### PAPER I

#### **REVIEW ARTICLE**

#### **PHOTOPERIODIC SYNCHRONIZATION AND DEVELOPMENTAL REGULATION OF THE ANNUAL REPRODUCTIVE CYCLE IN SALMONIDS**

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#### **In preparation**

**Contributions:** The present manuscript was compiled and written in full by the author of this thesis with advice and thorough proofreading by the co-authors.

**Keywords:** Atlantic salmon *Salmo salar* L., reproductive cycle, photoperiod, sexual maturation, developmental thresholds.

**Note:** Letters of references “Leclercq et al., 2010a, b, c” are not alphabetically in the thesis format of this manuscript (MS) due to cross-reference in other published MS within the present thesis.

**Abstract**

Annual photoperiod is recognized as the key environmental “zeitgeber” synchronizing the endogenous reproductive cycle of salmonids to the annual calendar-time. This provides a precisely time-defined spawning-window critical for offspring survival in temperate ecosystems. Reproductive success is also dependent on the genitor ability to support the energetic cost of reproduction such that the organism developmental and nutritional statuses are key determinants of sexual maturation.

This review aims at providing a conceptual framework of the overarching reproductive strategy of salmonids by focussing on the photoperiodic synchronisation and developmental regulation of the annual reproductive cycle in candidate first-time spawners. Addressed first is evidence of sexual development in autumn/early winter that correlates with body growth when the acquisition of nutritional and gonadal resources is critical for further continuation. This corresponds to the open-stage of the endogenous reproductive cycle including both a photo-neutral up to and a photo-responsive from around the winter solstice. This is shown by work on photoperiod manipulation demonstrating that exposure to continuous artificial-light (LL) decreases the population maturation rate only when applied after the winter solstice. The actual occurrence of long-days within the photo-responsive window translates into either gonadal photo-stimulation or photo-inhibition according to the fish developmental status that is assessed against genetically predetermined thresholds. The onset of differential photo-responsiveness, brought about by long-days, closes the open-stage of the endogenous reproductive cycle. It constitutes the “spring decision window” corresponding to a negative decision taken by individuals remaining immature such that salmonids population are clearly bimodal in term of sexual development under long-days. Immature fish (previously photo-inhibited) return to a photo-neutral stage at

the switch from long to short days and this allows resumption of gonadal activity marking initiation of the next reproductive cycle. Within the sexually recruited cohort it is hypothesized, based on evidence from non-salmonid species, that the transition to a short-day window brings about a period of photo-refractoriness ahead of the return to photo-neutrality. This would initiate gonadal resorption before completion if the level of gonadal development achieved so far is too poor. Such failed maturation events are discussed as a critical safety mechanism for the species propagation.

## 1. Introduction

Salmonidae are widely dispersed throughout temperate and polar latitudes and include a variety of species of strong ecological and economic interest. Among salmonidae, anadromous salmonids are particularly iconic due to their remarkable life-cycle culminating in a seasonal migration to their native river system for reproduction, i.e. homing. Sexual maturation in these species is a long-lasting and highly energetic process involving nuptial metamorphosis, upstream migration, active breeding behaviour (e.g. nesting, courtship, competition), gonadal development (Fleming, 1996; Hendry and Beall, 2004) and anorexic behavior at an advanced stage of sexual development (Kadri *et al.*, 1997b; Scott *et al.*, 2006). A total breeding cost of 50% to 70% of the total organism energy store is commonly reported in wild salmonids (Fleming, 1996; Hendry and Berg, 1999). This aforementioned breeding cost is likely to also reflect the anorexic behaviour of maturing fish and is overall responsible for a depletion of somatic resources (Aksnes *et al.*, 1986; Martin *et al.*, 1993; Bjerkeng *et al.*, 2000; Leclercq *et al.*, 2010b), accelerated senescence and a high probability of mortality. This is also true in iteroparous salmonids reproducing more than once in their life-time (Jonsson *et al.*, 1997; Hendry and Beall, 2004).

In contrast to the asynchronous ovarian type, where yolk accumulation varies with immediate food availability, vitellogenesis is primarily dependant upon body reserves in both semelparous and iteroparous salmonids exhibiting synchronous and group-synchronous ovarian development respectively (Murua and Saborido-Rey, 2003). In this later type, two to three oocyte cohorts occur simultaneously (Wallace and Selman, 1981; Tyler and Sumpter, 1996). The first cohort consists of oogonia undergoing primary oocyte growth and the second of oocytes at an endogenous vitellogenic stage. This cohort constitutes a “reserve fund” of pre-vitellogenic oocytes from which an oocyte group is recruited to undergo exogenous vitellogenesis in close synchrony. Initiation of exogeneous vitellogenesis indicates commitment toward maturation as this leading oocyte “clutch” ultimately constitutes the current-season batch of eggs shed within a few weeks in late autumn or early winter according to the species (Bromage and Cumaranatunga, 1988; Bromage *et al.*, 1992; Murua and Saborido-Rey, 2003).

This highly restricted spawning window is an adaptative trait inherent to the strong-calendar restriction inherent to temperate ecosystems so as to ensure fry emergence at the most suitable time for survival, i.e. spring (Sumpter, 1990; Migaud *et al.*, 2010). Seasonal variations that occur at all levels in temperate and polar ecosystems are arguably driven primarily by the annual photoperiod. Evolutionary persistent and unambiguous, seasonally changing day-length is recognized as the key environmental “zeitgeber” that synchronizes the endogenous reproductive cycle of seasonal breeders, such as salmonids, to the annual calendar-time (MacQuarriet *et al.*, 1979; Bromage and Duston; 1986; Randall *et al.*, 1998; Bromage *et al.*, 2001). Studies on both photoperiodic and developmental/nutritional determinants have advanced our understanding of how together they regulate sexual maturation in salmonids (Taranger

*et al.*, 2010). The gating model states that the endogenous reproductive cycle is on an open-phase under short-days such that sexual development progress and commitment toward maturation is allowed if the organism exceeds certain developmental thresholds (Duston & Bromage 1988). An analogous model is that of critical decision windows for initiation of early gonadal activity in autumn and continuation in the spring usually referred to as the “spring decision window”. In that model, both decisions would be based on the organism energetic resources being assessed physiologically and against genetically predetermined thresholds that are required to proceed (Rowe and Thorpe, 1990; Thorpe, 1994; Thorpe *et al.*, 1998; Campbell *et al.*, 2006).

From a wider perspective on teleost reproductive strategies, two interesting phenomena have been documented in teleosts but have remained overlooked so far: a seasonal cycle of gonadal activity that can be observed in immature fish before their first-spawn and the occurrence of failed or “dummy-run” maturation in pubertal teleosts. As compared to established spawners, first-time spawners have typically poorer reproductive performance along with a late and shorter spawning-window (Eliassen and Vahl, 1982; Bromage *et al.*, 1992; Bromley *et al.*, 1986; Bromley, 2000). The emission of gametes in insufficient quantity, quality or outside the spawning-window would compromise the viability of both the genitor and its progeny but commitment toward maturation nonetheless appears highly predictive in salmonids as it occurs months before the spawning window. Evolutionary mechanisms are therefore likely to be in place to allow for a tactical change in the reproductive strategy of the poorer performers. This would consist in interrupting gonadogenesis and redirect to the soma the resources invested up to that point in the gonads.

Both phenomena of gonadal activity in immature fish and dummy-run (aborted) maturation could provide significant insight into the reproductive strategy of salmonids

and its annual entrainment. The autumn decision window for initiation of early gonadal activity is in opposition to the gating model where the endogenous reproductive cycle is said to be open under short-days. While proof of gonadal activity under short-days exists, few studies provide evidence of commitment into sexual maturation prior to the onset of long-days as should be expected from the gating model. Finally, the critical “spring decision window” suggest that the decision to mature is taken at, or following, the occurrence of long-days which appears contradictory to the gating model. Despite decades of studies on the photoperiodic manipulation of reproduction, the working hypothesis on the photoperiodic regulation of gonadal activity remains ambiguous in salmonids unlike in avian species (Sharp, 1996; Hahn and MacDougall-Shackleton, 2007). Similarly, the photo-neuro-endocrine system (PNES) through which the photoperiod regulates the reproductive cascade in relation to developmental and nutritional factors remains poorly understood. Driven by work on other vertebrates, an increasing number of neuro-endocrine factors are recognized as permissive or central to the functionality of the PNES that connects photic stimulation to the brain-pituitary-gonad (BPG) axis (Migaud *et al.*, 2010).

A conceptual framework of the annual photoperiodic entrainment and developmental regulation of salmonid reproduction is likely to facilitate unveiling the organisation of the PNES system in salmonids in relation to the BPG and somatic-axis. This is ultimately required to validate the overarching reproductive strategy of salmonids and can be considered as a prerequisite to characterize the effect of more secondary determinants of sexual development such as environmental (e.g. temperature, salinity), physiological (e.g. stress) and behavioural (e.g. pheromones) parameters. In practice, a better knowledge of salmonid reproductive strategy is key to improving their

conservation under increasing environmental pressure, their propagation in various environmental conditions and their somatic growth in the aquacultural industry.

The photoperiodic synchronisation and developmental regulation of the salmonid reproductive cycles is reviewed in this paper with the aim of providing an understanding of their overarching reproductive strategy within the annual framework. To do so, the concepts of photo-neutrality, -responsiveness, -inhibition, -stimulation and -refractoriness used in avian species are applied and the “gating” and “critical window” models jointly incorporated. First addressed is the photo-neutral window of the endogenous reproductive cycle under a short or decreasing day-length when gonadal activity exist and the acquisition of both nutritional and gonadal resources is critical for further continuation. Evidences of a switch to photo-sensitivity around the winter solstice are addressed and shown to translate into differential photo-responsiveness under long-days (photo-inhibition or photo-stimulation) which closes the open-gate of the endogenous reproductive cycle. Finally and based on non-salmonid species, the existence of a critical “confirmation” window is hypothesized as corresponding to a period of photo-refractoriness following photo-stimulation in the sexually maturing individuals. Although it is beyond the scope of this work to explore the PNES and somatic/nutritional factors involved in sexual maturation, recent knowledge and key factors are summarized in order to highlight directions for future investigations.

## **2. The open-phase of the reproductive cycle**

### **2.1. Occurrence of gonadal activity in autumn/winter a year prior to completion**

In rainbow trout, *Oncorhynchus mykiss* (Bromage, 1995) and brown trout, *Salmo trutta* (Billard, 1987) established-spawners, mitotic division of oogonia occurred immediately after spawning in autumn. In virgin female rainbow trout spawning in January,

hormonal events controlling reproduction initiated at least 1 year before first ovulation when a significant proportion of oocytes at the endogenous vitellogenesis stage were observed (Sumpter *et al.*, 1984). In virgin female Atlantic salmon, *Salmo salar*, it was concluded, based on plasma oestradiol-17 $\beta$  (E<sub>2</sub>)-levels that sexual maturation commenced in most females by early January prior to the long-day onset (Taranger *et al.*, 1999). More recently in this species, secondary oocyte-growth was said to start with rising plasma E<sub>2</sub> in the autumn, at least a year prior to completion, followed by entry into true vitellogenesis around the winter solstice (Taranger *et al.*, 2010). This is in accordance with findings in Atlantic salmon parr where the annual cycle of gonadal growth starts in autumn as shown by a GSI increasing from November to April (Crim and Evans, 1978; Dodd *et al.*, 1978; Thorpe *et al.*, 1990). It was reported in this species that plasma 11-oxotestosterone and 11-ketotestosterone (11-KT), known to stimulate early mitotic division of germ cells, rise 10-11 months prior to spermiation in December-January (Hunt *et al.*, 1982; Youngson *et al.*, 1988; Miura *et al.*, 1991; Stead *et al.*, 1999). In virgin male spring Chinook salmon, *Oncorhynchus tshawytscha* (September spermiation), the mitotic proliferation of primary A-spermatogonia and a decrease in the ratio of primary A- to transitional-spermatogonia were observed from July to December 14-months prior to completion and in correspondance with a rise in plasma 11-KT, pituitary FSH and GSI in the whole population (Campbell *et al.*, 2003). In this study, plasma 11-KT level was bimodal a year prior to completion and the upper mode reflected, in January, the final population maturation rate observed over the spawning window. This highlighted a likely commitment toward maturation under short-days and confirmed previous findings in this species where histological and physiological evidence of sexual development were observed in November nearly 1-year prior to spermiation (Shearer and Swanson, 2000).



The most comprehensive evidence of gonadal activity in the fall a year prior to completion was documented in female coho salmon, *Oncorhynchus kisutch*, in the form of increasing plasma FSH and E<sub>2</sub> levels, oocyte diameter and GSI from cortical alveoli accumulation and expression of key ovarian genes (Campbell *et al.*, 2006). Recently, initiation of exogenous vitellogenesis, indicating commitment toward maturation, was observed at an increasing rate from October to November in 2-SW female Atlantic salmon (Leclercq *et al.*, 2010c). Entry into vitellogenesis was not previously reported before the winter solstice, but suggested to occur in December (Taranger *et al.*, 2010), likely due to the comparatively lower developmental status of the the 1-SW age-class that are more commonly studied.

As explained by the gating model (Duston and Bromage, 1988), this is strong evidence that the short-day period corresponds to an open-phase in the reproductive cycle of salmonids when gonadal activity occurs and recruitment into sexual maturation can proceed in the most advanced individuals.

## **2.2. The importance of energetic resources acquired during the open-stage**

In salmonids, the energetic cost of reproduction associated with gonadal investment, anadromous migration, nuptial metamorphosis and breeding behaviour appears fuelled by resources previously accumulated in the soma (Hendry and Berg, 1999; Silverstein *et al.*, 1999). The importance of somatic resources is highlighted by the anorexic behaviour of fish at an advanced stage of gonadogenesis (Kadri *et al.*, 1997b; Scott *et al.*, 2006) and is overall illustrated by the significant deterioration of flesh quality suffered by salmonids undergoing sexual maturation (Aksnes *et al.*, 1986; Martin *et al.*, 1993; Bjerkeng *et al.*, 2000; Johnston *et al.*, 2006; Leclercq *et al.*, 2010b). In order to meet the energetic requirements of reproduction, salmonids at an early stage

of sexual maturation have an accelerated growth rate and accumulate large lipid deposits (Bell *et al.*, 1998; Kadri *et al.*, 1996, 1997a). This “anabolic window” is consecutive hence follows recruitment into sexual maturation. Although not always clearly distinguishable, the energetic/developmental resources previously accumulated also appear to act as determinants (causative) of initiation of sexual maturation.

A strong relationship between somatic resources and the age of entry into puberty has been reported in various salmonids (Taranger *et al.*, 2010). This is responsible for the earlier onset of puberty in farmed populations (Fleming, 1996; Thorpe *et al.*, 1998; Taranger *et al.*, 2010) and also in heavily exploited wild populations where younger, smaller fish would thrive on a reduced competition for nutritional resources from older, bigger, conspecifics (Thorpe, 2007). Ultimately linked, the respective roles of body size, energy stores and growth rate as determinant of maturation remain poorly distinguished (Shearer and Swanson, 2000). It was suggested in small male Chinook salmon that body fat levels were more important than growth rate or size as the prime somatic determinant in bigger individuals (Silverstein *et al.*, 1998; Shearer *et al.*, 2006). In semelparous sockeye salmon, *Oncorhynchus nerka*, stored fat is mainly used for anadromous migration and egg production while proteins are recruited at a later stage to support nuptial metamorphosis and metabolism during spawning (Hendry and Berg, 1999). In this study, somatic fat decreased by 76% and protein by 12% from freshwater entry to the start of spawning. Lipids are the most concentrated energy source and can be readily mobilized while proteins have an important role in supporting body structure and activity (Love, 1980; Jobling, 1994; Hendry and Berg, 1999). Lipids are generally considered as the most decisive somatic resources to initiate and sustain the reproductive effort in salmonids (Thorpe *et al.*, 1994; 1998; 2007).

Feed deprivation studies highlight the importance of somatic resources early in the reproductive cycle, during the open-phase of the reproductive cycle. Feeding rainbow trout a low ration from 12 to 8 months prior to the spawning window (in December-January) had the most significant effect on reducing the population maturation rate, but also affected individuals' fecundity which would be established early in the reproductive cycle (Bromage *et al.*, 1992). In accordance, a clear correlation was observed between the maturation rate of 1+ male Chinook salmon and their size and adiposity a year earlier in autumn/winter when maturation was initiated (Silverstein *et al.*, 1998; Shearer and Swanson, 2000). In Atlantic salmon, feed deprivation in autumn (November-December) compared to late winter/spring (February-April) had a more pronounced effect on reducing grilising rate, although deprivation over both periods was the most effective (Duston and Saunders, 1999). In other studies, food restriction in February-March or spring when intake normally increases was more effective at inhibiting maturation in both sexes as compared to December-January or winter (Rowe and Thorpe, 1990; Thorpe *et al.*, 1990). Conversely, feed deprivation in May/June only marginally reduced Atlantic salmon maturation such that low growth or poor condition at this time do not necessarily prevent gonadal development (Berglund; 1995). Closer to the spawning window, restricted feed ration would have a reduced effect on salmonid maturation as further shown in rainbow trout where low feeding for 3 months immediately prior to the spawning window had no effect on stock with a previous history of high feeding level (Bromage *et al.*, 1992).

The strong effect of food restriction in winter/early spring ahead of the long-day cue was previously discussed as an adaptative mechanism preventing maturation if sufficient energetic reserves have not been acquired (Rowe and Thorpe, 1990; Reimers *et al.*, 1993).

### **2.3. The importance of gonadal development during the open-stage**

As previously hypothesised (Sutterlin and McLean, 1984; Chadwick *et al.*, 1986), “maturation may also depend on the acquisition of a certain stage of gonadal development prior to a critical time in winter/spring, as well as the need to exceed some growth related threshold” (Taranger *et al.*, 1999). Bromage *et al.*, (1992) suggested that changes in fecundity and maturation rate induced by a lower feeding regime occurred through an alteration of the recruitment of pre-vitellogenic oocytes into vitellogenesis. Similarly in farmed Atlantic cod, oocyte recruitment into previtellogenic oocytes was a key determinant of fecundity that correlated with the fish nutritional status (Kjesbu and Klungsøyr, 1991). Female winter flounder was also shown to have a nutritionally sensitive window for early gametogenesis when starvation led to an arrestment of oocyte development at the previtellogenic stage (Burton, 1994). More recently in coho salmon, body growth in autumn was shown to positively correlate with previtellogenic ovarian growth and this time and with the degree of oocyte development the following spring (Campbell *et al.*, 2006). It is accordingly not surprising that a reduction in feed intake during the open-stage of the reproductive cycle decreases potential fecundity and oocyte growth until a point where maturation will be postponed to the following year (Knox *et al.*, 1988; Bromage *et al.*, 1992).

### **2.4. The photo-neutral and photo-responsive stage within the open-phase**

The open-phase of the reproductive cycle would incorporate both a photo-neutral and a photo-responsive stage as shown by work on photoperiod manipulation. Exposure to artificial continuous-light (LL) prior to the winter solstice typically either did not affect or increased the population maturation rate as compared to a natural

photoperiod (NL) or to LL-exposure after the winter solstice (Endal *et al.*, 2000; Johnston, 2003; Oppedal *et al.*, 2003). Known to stimulate appetite and growth in salmonids (Saunders and Harmon, 1988; Oppedal *et al.*, 1997; 2006; Hansen *et al.*, 1992; Endal *et al.*, 2000; Taylor *et al.*, 2005, 2006; 2007), LL applied during the photo-neutral window is likely to enhance gonadal activity indirectly through its positive nutritional effect ultimately increasing the population maturation rate. Conversely when applied shortly after the winter solstice, the switch from short- to long-days brought about by LL significantly reduces the maturation rate of sea-reared Atlantic salmon (e.g. Duston and Bromage, 1987, 1988; Taranger *et al.*, 1998, 1999; Bromage *et al.*, 2001; Unwin *et al.*, 2005) as further described later. The varying potency of LL when applied before or after the winter solstice demonstrates that the organism's endogenous reproductive cycle becomes responsive to long-days after a minimum period of exposure to short-days, circa the winter solstice, defining a shift from a photo-neutral to a photo-responsive stage. Both those stages together constitute the open-phase of the reproductive cycle that is later closed by the natural or artificial occurrence of long-days (Duston and Bromage, 1988).

### **3. Differential photo-responsiveness and return to photo-neutrality**

#### **3.1. Long-days close the open-phase of the reproductive cycle**

Photoperiodic studies previously mentioned led to the recognized hypothesis of a critical "spring decision window" at the shift from short-to-long days during the photo-responsive stage. At that stage only, long-days would close the open-phase of the salmonid reproductive cycle such that sexual development would proceed further only in organisms exceeding predetermined developmental thresholds (Rowe and Thorpe, 1990; Thorpe, 1994; Thorpe *et al.*, 1998; Taranger *et al.*, 1999; Campbell *et al.*, 2003;

2006). By prematurely closing the open-phase for gonadal activity, LL-applied after the winter solstice, but before the naturally occurring long-days, reduces the maturation rate of farmed salmonid populations (McCormick and Naiman, 1984; Duston and Bromage, 1987, 1988; Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Taranger *et al.*, 1998, 1999; Porter *et al.*, 1999; Bromage *et al.*, 2001; Unwin *et al.*, 2005). Applying LL shortly after the winter solstice (marking the onset of the photo-responsive stage) is recognized as the most efficient in that aim and is routinely practiced in the Scottish salmon industry (Leclercq *et al.*, 2010a). A later switch to long days (e.g. March) is less effective as the organism's endogenous reproductive cycle remains responsive longer, thereby increasing the period of opportunity for the organism to exceed the required developmental thresholds (Björnsson *et al.*, 1994; Taranger *et al.*, 1998). Due to the high plasticity in age and size at first maturity observed between strains and families, the required developmental thresholds are considered to be genetically determined and to a degree adaptive to local environmental conditions in order to optimize reproductive success (Thorpe *et al.*, 1998, Taranger *et al.*, 2010).

### **3.2. Photo-stimulation and photo-inhibition segregates the maturity cohorts**

Importantly, the mechanism that closes the open-gate of the reproductive cycle and constitutes the “spring decision window” appears to be the onset of differential photo-responsiveness in the population: photo-stimulation or photo-inhibition of gonadal development according to the individual's developmental status.

As previously addressed, gonadal activity already exists and commitment toward maturation can occur during the open-phase under short-days. However, long-days are concomitant with a significant increase in GSI and plasma levels of reproductive hormones in both males and females and an active accumulation of

exogenous vitellogenin in maturing oocytes (Aknes *et al.*, 1986; Taranger, 1998; Unwin *et al.*, 2005). This photoperiodic cue was therefore said to stimulate the BPG-axis of the maturing cohort (Schulz *et al.*, 2006). Inversely, arrestment of oocyte development not beyond the oil drop stage and a decline in plasma sex steroids was observed following 4 to 6 weeks of LL exposure in Atlantic salmon remaining immature over the remaining of the reproductive season (Taranger *et al.*, 1998; 1999). Similarly in male Atlantic salmon, LL-applied after the winter solstice arrested testis development and reduced testis activity below the level observed prior to LL application (Schulz *et al.*, 2006). In these experiments, gonadal development and plasma sex-steroid levels remained basal in the immature fish over the whole period of LL-exposure from January to June. This is in accordance with the constant GSI reported in immature Chinook salmon between April and July under a natural photoperiod (increasing daylength) (Campbell, 2003).

These results together indicate that long-days elicit the decision *not* to mature by inducing photo-inhibition of the BPG-axis in the cohort remaining subsequently immature. Inversely, photo-stimulation of the maturing cohort accentuates the gonadal activity previously initiated during the open-phase. Maturation was indeed said to be regulated by inhibition rather than being “switched-on” in Atlantic salmon (Thorpe *et al.*, 1998). Photo-inhibition of gonadal development would favour somatic growth to reinforce the fitness and reproductive success of future genitors predicted as unable to complete maturation within the forthcoming reproductive cycle.

### **3.3. Closing the annual reproductive cycle: Long-to-short photoperiod manipulation**

Due to the preponderant effect on salmonid maturation of the “short-to-long” photoperiodic cue, few studies have specifically addressed the effect of a long-to-short

day signal (Schulz *et al.*, 2006). It was recently shown in Atlantic salmon that, following LL-onset in January (9-month post seawater transfer), LL-offset at different times before the summer solstice (April, May or June) had no effect on the maturation rate of the population harvested over the subsequent autumn (Leclercq *et al.*, 2010c). In this study, the GSI of immature fish was however higher in October and in November than in June at the time of LL-offset. More importantly, the GSI and proportion of females initiating true vitellogenesis (primary yolk stage but immature in commercial term) marking the onset of the next reproductive cycle was higher in stocks returned to NL earlier (April as compared to June). In Arctic charr, *Salvelinus alpinus*, and rainbow trout an earlier shift to short-days following LL-onset in January advanced and synchronized spawning, decreased gamete quality and did not affect or reduced the population maturation rate (Duston and Bromage, 1988; Duston *et al.*, 2003; Frantzen *et al.*, 2004). In these experiments, the effect of photoperiod treatment on the immature cohort was not specifically addressed. Importantly, the reduction in maturation rate was suggested, as in short-to-long photoperiod manipulation, to be due to fewer fish meeting required developmental thresholds at this critical long-to-short day signal. This photoperiodic signal could therefore constitute a critical cue within the annual reproductive cycle.

In the immature cohort previously photo-inhibited, return to short-days appears to allow resumption of gonadal activity marking the initiation of the next-reproductive cycle and this highlight a return to photo-neutrality to (Leclercq *et al.*, 2010c). In the sexually maturing cohort that was previously photo-stimulated, this photoperiodic cue is suggested to induce a period of photo-refractoriness. At this stage, maturing fish that do not comply with a threshold level of gonadal development or hold excessively depleted energetic resources would not attempt to complete gonadogenesis but redirect to the



soma the resources invested so far in the gonads as discussed. Conversely, satisfactory energetic reserves and/or level of gonadal development, possibly providing sufficient auto/paracrine gonadal stimulation, would override the photo-refractoriness as to allow continuation of gonadogenesis for completion by the fitter reproducers only.

#### **4. Evidence of a confirmation window from non-salmonid species**

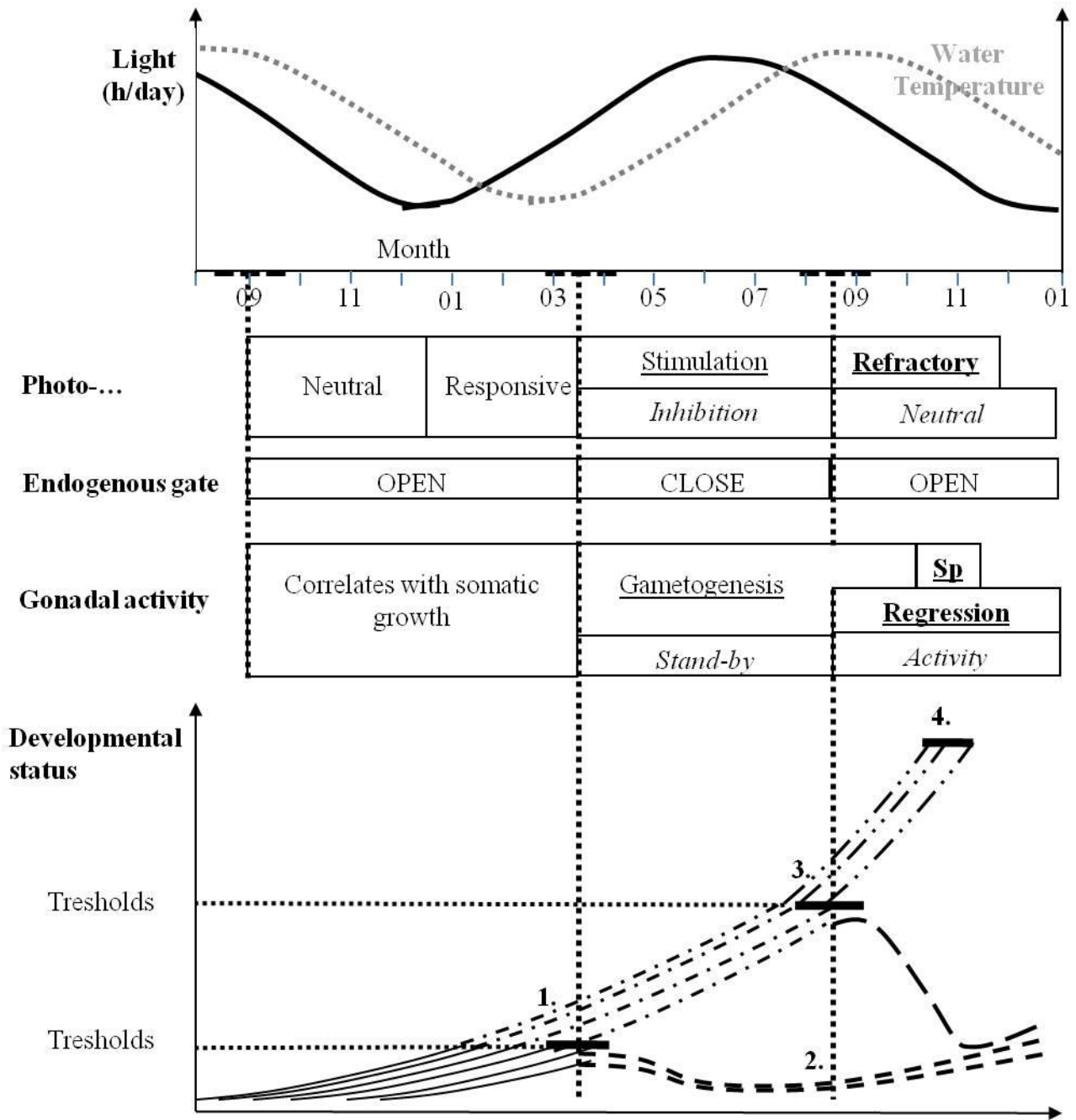
##### **4.1. The avian model for photo-refractoriness**

In avian species it is recognized that coincident with photo-stimulation is the building-up of a photo-inhibitory effect of long-days which eventually leads to a point where the inhibition overrides the stimulation, a net effect referred to as photo-refractoriness (Sharp, 1996; Hahn and MacDougall-Shackleton, 2007). The switch on a dominant inhibition can be reached before the summer solstice (absolute refractoriness) or following a decrease in day-length (relative refractoriness) depending on genetically determined factors and is key in the timing of egg laying and gonadal regression. Later in autumn, the reduction in day-length would eventually dissipate the photo-refractoriness returning the organism to a photo-neutral or photo-responsive stage where gonadal growth is allowed (Sharp, 1996; Hahn and MacDougall-Shackleton, 2007).

##### **4.2. A confirmation window required**

Timed in spring for autumn/winter spawning salmonids, the direction taken at the onset of differential photo-responsiveness (photo-inhibition or photo-stimulation) appears highly predictive. First-time compared to established spawners have a reduced total fecundity but a similar relative fecundity (Eliassen and Vahl, 1982), produce smaller eggs following a reduced oocyte growth (Bromage et al, 1992) and have a reduced and late spawning window in the wild (Bromley *et al*, 1986; Arnold and

Metcalf, 1995; Bromley, 2000). Characterized by poorer reproductive performance, candidate first-time spawners are high-risk breeders hence a critical generation for species propagation. In particular, the need to achieve high reproductive success is clearly critical in semelparous salmonids reproducing once their life-time. The onset of photo-refractoriness could therefore constitute a safety mechanism, a confirmation window where a reduced quantity/quality of gametes or a depleted soma would reveal the weakness of the reproductive outcome and/or the inability to complete maturation at the spawning window. By permitting a tactical change in the reproductive strategy of individuals undergoing a weak reproductive cycle, this check-point would also allow for a more opportunistic continuation of gonadal development at the onset of differential photo-responsiveness.



**Figure 1.** Schematic representation of salmonid annual reproductive strategy as entrained by the annual photoperiod rhythm and regulated by developmental thresholds.

Early sexual development was reported in the fall a year before completion in various salmonids (solid lines) (e.g. Sumpter *et al.*, 1984; Shearer and Swanson, 2000). At this stage, it correlates with body-growth and recruitment into sexual maturation could proceed in the most advanced individuals (Campbell *et al.*, 2003; Leclercq *et al.*, 2010c). The short-day photoperiod window would therefore correspond to an open-phase in the reproductive cycle of salmonids (the gating model, Duston and Bromage, 1988). This open-phase would further incorporate both a photo-neutral and a photo-responsive stage as shown by the fact that salmonid populations are responsive to a

long-day photoperiodic signal from the winter solstice only (Taranger *et al.*, 1998, 1999; Endal *et al.*, 2000; Oppedal *et al.*, 2003). **1.** The transition to long-days constitutes, when occurring, the “spring decision window” by closing the open-phase of the reproductive cycle such that sexual development would continue further only in organisms exceeding predetermined developmental (nutritional/somatic and gonadal) thresholds (e.g. Rowe and Thorpe, 1990; Thorpe, 1994; Thorpe *et al.*, 1998; Campbell *et al.*, 2006). Salmonid populations are clearly bi-modal in term of sexual development under long-days inherent to a photo-stimulation of gonadogenesis in fish exceeding predetermined developmental thresholds (dash-dot lines) and a photo-inhibition of gonadal development in fish below these thresholds (dash lines) (Taranger *et al.*, 1999; Schulz *et al.*, 2006). The “spring decision window” is therefore a negative decision taken by the cohort remaining immature only. **2.** A decreasing or short-day cue photoperiod would return the immature cohort (dash lines) to a photoneutral phase so as to initiate the next-reproductive cycle (Leclercq *et al.*, 2010c). **3.** In the sexually maturing cohort (dash-dot lines), this photoperiodic cue can be hypothesized, based on non-salmonid evidence, as inducing a period of photo-refractoriness. At this stage, maturing fish that do not comply with a threshold level of energetic resources and/or gonadal development would interrupt gonadogenesis through intense atresia (long-dash lines) (Bromage *et al.*, 1992; Sharp *et al.*, 1996; Bromley *et al.*, 2000; Rideout *et al.*, 2005). This could constitute a critical safety mechanism, a “confirmation window” allowing gonadal resorption if the organism is deemed unable to complete gonadogenesis at the spawning window. Conversely, satisfactory energetic reserves and/or level of gonadal development would override the photo-refractoriness, possibly through sufficient auto/paracrine gonadal stimulation, so as to allow continuation of gonadogenesis for completion by the fitter reproducers only (long dash-dot-dot lines). **4.** Spawning window (**Sp.**). Words in italic and dash lines specifically refer to the immature cohort. Words underline and dash-dot lines specifically refer to the sexually recruited cohort. Words in bold and underline refers to successful reproducers (refractory, Sp.; long dash-dot-dot line) or failed first-time spawners (refractory, regression; long-dash lines).

### 4.3. Gonadal evidence: dummy-run maturation, atresia and nutrition

Despite the common assumption that all fish can be clearly classified as mature or immature during the spawning season, a number of studies report evidence of aborted maturation within the recruiting year class. Such failed reproductive events were characterized by initiation of vitellogenesis followed by intense atresia leading to oocyte resorption towards the end of the reproductive season as observed in rainbow trout (Elliot *et al.*, 1983); sole, *Solea solea* (De Veen, 1970; Ramsay, 1993; Ramsay and Witthames, 1996) , plaice, *Pleuronectes platessa* (Bromley, 2000), flounder, *Liopsetta obscura* (Yamamoto, 1956), Atlantic halibut, *Hippoglossus hippoglossus* (Björnsson et

al, 1998), hake, *Merluccius merluccius* (Hickling, 1935), catfish, *Tandanus tandanus* (Davies, 1977), white grouper, *Epinephelus aeneus*, (Hassin *et al.*, 1997) and black bullhead, *Ictalurus melas* (Dennison and Bulkley, 1972). Those individuals have been variously defined as adolescent (Hickling, 1930; Dennison and Bulkley, 1972), undergoing a tentative pre-spawning maturation (Yamamoto, 1956), an interrupted ovarian development (De Veen, 1970) or a “dummy-run” (Davis, 1977; Eliassen and Vahl, 1982; Hassin *et al.*, 1997).

Atresia is common in virtually all fish species and usually occurs within vitellogenic oocytes (Kjesbu *et al.*, 1991; Bromage *et al.*, 1992; Witthames and Greer Walker, 1995). Under normal circumstances less than 10% of the vitellogenic oocytes become atretic (Bromage and Cumarantunga, 1987, 1988) and this may provide the “fine-tuning” of fecundity in the later stages of the reproductive cycle (Bromage *et al.*, 1992). As previously addressed, restricted feeding for 3 months prior to spawning had the smallest impact on rainbow trout maturation (compared to restriction later in the cycle; Bromage *et al.*, 1992) but extreme conditions such as starvation can lead to resorption of all vitellogenic oocytes through atresia (Bromage and Cumarantunga, 1987, 1988). Similarly in Atlantic cod, atresia was inversely related to nutritional status (Kjesbu *et al.*, 1991) while in first-time spawning turbot, *Scophthalmus maximus*, low ration during vitellogenesis resulted in low growth of vitellogenic oocytes and total resorption in a third of the females (Bromley *et al.*, 2000). Complete resorption of vitellogenic oocytes suggests that biological mechanisms exist to prevent further gonadal investment when the organism’s soma is at risk. In salmonids known to be anorexic or to feed poorly during their anadromous migration, the depletion of somatic reserves could be checked at the photo-refractory period and be responsible for gonadal resorption through atresia.

Rideout *et al.* (2005) recognized the frequent occurrence of skipped spawning through resorption of vitellogenic oocytes in female iteroparous fish as the “reabsorbing” spawning omission type. They reviewed that skipped spawning also occurs in established spawners with another two types of skipped spawning events: “resting” (no initiation) and “retaining” (no spawning), all of which were most often related to poor nutritional conditions.

#### **4.4. Behavioural evidences: dummy-run migration**

Wild stock surveys provide evidence of “dummy-run” spawning migration that did actually not culminate in spawning in immature plaice (Arnold and Metcalfe, 1995) and cod (Woodhead, 1959; Rose, 1993). Immature cod of four and five years-old have been shown to join the migrating school (Rose, 1993) during a false spawning migration that increases in length as the immature fish get older and bigger (Trout, 1957, Woodhead, 1959). Arnold and Metcalfe (1995) also observed immature female plaice migrating toward the spawning ground late in the spawning season and coming back towards the feeding area soon after. Their study also showed a tendency for larger/older females to migrate and spawn first and to be progressively replaced by younger plaice. Interestingly but with limited evidence, both dummy-run migration and maturation were observed in immature fish approaching the age and body-size of conspecific first-time spawners. Gonadal maturation and migration behaviour are triggered and controlled in parallel by the same neuro-hormonal system i.e. the BPG axis (Scott *et al.*, 2006). Both phenomena are likely to be related to the photo-stimulation of the BPG axis subsequently interrupted through photo-refraction.

#### **4.5. An interrupted puberty following life-long and seasonal gonadal activity**

The phenomenon of dummy-activation of the reproductive-axis in first-time spawners is reinforced by the observation of an annual GSI cycle in various immature teleosts that mimics that observed in mature fish. Miniaturized seasonal cycles of gonadal development were observed around the opening of the reproductive period in both sexes of catfish (Davis, 1977), cod (Eliassen and Vahl; 1982) and 2 species of grouper (Bruslé and Bruslé, 1976). In Atlantic salmon, although remaining low, the GSI of immature parr significantly rose from November to April and was significantly lower in July and August (Crim and Evans, 1978; Dodd *et al.*, 1978). A seasonal variation in liver mean weight was also detected in the immature hake, being lighter in August, despite no signs of on-going vitellogenesis (Hickling, 1930). More extensive studies were conducted on immature hake and garfish. From their first to third year, an increase in the maximum ovum diameter in immature garfish was observed during the spawning season. Annual ova enlargement was “beyond that shown the previous year”, remained below the ova diameter of first time-spawners and was followed by regression in early winter (Ling, 1958). In immature hake, years before reproduction, the process of synapsis was more active in winter months, increasing the general oogonia-stock and decreasing the mean oocyte-diameter following a maximum in summer (Hickling, 1935). Incipient formation of oil droplets was observed in the biggest oocytes from the fourth year, a process that progressively increased along with oocyte diameter until the ninth year. At this time, fully mature oocytes were observed but resorbed at the close of the reproductive season before successful reproduction the following year. Later in established-breeders, fecundity and egg size typically increases progressively while the spawning ground is also reached earlier in older breeders (Eliassen and Vahl, 1982; Arnold and Metcalfe; 1995; Bromage *et al.*, 1992; Bromley, 2000).

In this seasonal pattern of gonadal activity coupled to a life-long increase in reproductive power, the phenomenon of dummy-run maturation appears as an intermediary cycle between the ones observed in immature and successful first-time spawners. It can be considered as an interrupted puberty or aborted maturation in candidate first-time spawners that reveals unable to complete reproduction and this might occur through the photo-refraction of the BPG-axis following its opportunistic photo-stimulation.

## **5. Overview of the main photo-neuro-endocrine factors involved**

It is beyond the scope of this work to review the recently developing knowledge on the photo-neuro-endocrine system (PNES) regulating reproductive events in interaction with nutritional messengers. Driven by work in mammals, an increasing number of factors are rapidly emerging in teleosts but mechanisms remain overall unclear (Migaud *et al.*, 2010). Two recent works reviewed the regulation of the reproductive-axis in teleosts. Zohar *et al.* (2010) described the neuroendocrine pathways constituting the BPG-axis while Migaud *et al.* (2010) addressed, in particular, mechanisms through which photoperiod is perceived and regulates reproductive events.

### **5.1. Photoperiod perception: Melatonin**

The hormone melatonin released by the pineal gland is now widely recognized as the key time-keeping hormone that accurately reflects the perceived photoperiod in teleosts as in other vertebrates (Bromage *et al.*, 2001; Migaud *et al.*, 2006; Falcon *et al.*, 2010). More specifically, the activity of the key regulatory enzyme in the melatonin biosynthetic pathway (arylalkylamine *N*-acetyltransferase; AANAT) was shown to be regulated by photo-induced molecular events responsible for the circadian rhythm in



plasma melatonin level (Begay *et al.*, 1998; Falcón *et al.*, 2010; Migaud *et al.*, 2010). Although a key role of melatonin in the transduction of photoperiodic signals to the brain-pituitary-gonadal (BPG) axis was hypothesized in salmonids (Mayer *et al.*, 1997; Amano *et al.*, 2000), evidence for its direct involvement in the entrainment of teleost reproductive cycles is still lacking. It is now increasingly accepted that melatonin *per se* is an unlikely gatekeeper of reproduction in teleosts as in other vertebrates (Migaud *et al.*, 2010).

## 5.2. Candidate factors for initiation of puberty through photo-transduction

In the last decade, two new factors have emerged at the interface between environmental, metabolic signals and the BPG-axis. The KiSS system, made of the protein receptor 54 (GPR54) and its ligands the kisspeptins, is increasingly considered as a key catalyst and regulator of reproduction in mammals (Tena-Sempere, 2006; Revel *et al.*, 2007; Smith and Clarke, 2007). Interestingly, the activation of the KiSS-system would be mediated by melatonin signaling events modulating KiSS gene expression and indirectly sex-steroid sensitivity (Grieves *et al.*, 2008). Comparatively poorly documented, current evidence confirm the importance of this novel pathway in fish. This includes the co-localization of GPR54 and gonadotropin-releasing hormone (GnRH) systems (Parhar *et al.*; 2004; Kitahashi *et al.*, 2009; Filby *et al.*, 2008), the higher expression of GPR54 at an early stage of gonadal development (Mohamed *et al.*, 2007; Nocillado *et al.*, 2007; Biran *et al.*, 2008; Martinez-Chavez *et al.*, 2008) and the sensitivity of kisspeptin-neurons to sex steroid feedback (Kanda *et al.*, 2008). The prevalent hypothesis is that kisspeptin would positively regulate GnRH neurons hence gonadotropin secretion, as observed in European sea bass (*Dicentrarchus labrax*) where *in vivo* administration of kisspeptines induced follicle stimulating hormone (FSH) and

Luteinizing Hormone (LH) secretion (Felip *et al.*, 2009). However, the link between the photic information and stimulation of the kisspeptin-system remains unclear (Migaud *et al.*, 2010).

Developing knowledge on the regulation of thyroid-stimulating hormone (TSH) and thyroid-hormone-activating enzyme (type 2 deiodinase, Dio2), that catalyse the intracellular deiodination of thyroxine (T4) prohormone to the active triiodothyronine (T3), is promising in linking photoperiodic signals to reproductive events. The gene expression of Dio2 in the mediobasal hypothalamus was indeed stimulated by the light-induced production of thyroid-stimulating hormone (TSH) in the *pars tuberalis* (PT) of the pituitary gland where a high density of melatonin receptors is observed (Nakao *et al.*, 2008; Ono *et al.*, 2008). This constitutes the earliest photoperiodic signal transduction event yet documented which was shown, through administration of TSH, Dio2 inhibitor and T3 to trigger long-day photo-induced gonadal growth in a seasonal avian species (Yoshimura *et al.*, 2003; Nakao *et al.*, 2008; Ono *et al.*, 2008). Such photoinduced regulation of the thyroid-axis remains to be demonstrated in teleosts but its role in sexual development is well documented. Orozco *et al.* (2003) reviewed the correlations between T3 and reproductive status in fish, including salmonids, while demonstrating the particular role of hepatic T3 in female rainbow trout vitellogenesis and oogenesis.

These new factors in photoperiodic signal transduction are likely to ultimately help elucidate how energy homeostasis messengers interact with the reproductive-axis as determinants of its photo-sensitivity. Candidates include among others insulin-like growth factor (IGF), growth hormone (GH), somatolactin (SL), ghrelin and leptin (Weil *et al.*, 2003; Wood *et al.*, 2005; Yu and Ge, 2007; Benedet, 2008; Kaiya *et al.*, 2008; Migaud *et al.*, 2010; Zohar *et al.*, 2010).

### 5.3. Key messengers of somatic resources

The role of IGFs in fish reproduction was recently reviewed (Reinecke, 2010) and IGF1 is considered as a key messenger of metabolic status and growth to the reproductive-axis (Taylor *et al.* 2008; Shearer and Swanson 2000; Furukuma *et al.* 2008). It was suggested to have a main role in early primary oocyte growth (Campbell *et al.* 2006), increases gonadotropin synthesis at the previtellogenic oocyte stage (Baker *et al.*, 2000; Furukuma *et al.* 2008) and stimulates ovarian steroidogenesis as does GH and SL (Planas *et al.*, 1992; Le Gac *et al.*, 1993; Maestro *et al.*, 1997). In the spring, plasma IGF1 correlated with body-growth and hormonal factors of the BPG-axis in coho salmon (Campbell *et al.* 2006). Insulin was shown to stimulate oocyte multiplication and vitellogenin uptake through IGF-receptors in rainbow trout (Shibata *et al.*, 1993). More recently, evidence of a photoperiod-driven GH-IGF-gonad axis functional from January onward were provided in Atlantic salmon and shown to be inhibited by continuous-light (Benedet, 2008). It was further suggested that LL has different effects on GH dynamics in immature and maturing Atlantic salmon (Björnsson *et al.*, 1994; Benedet *et al.*, 2010).

Lipid resources are proposed as the most decisive nutritional factor determining reproductive events (Thorpe *et al.*, 1994, 2007). It is therefore not surprising that leptin, acting as an adipose signal and regulating energy balance, is a likely messenger of nutritional status to the reproductive axis and even a potential metabolic gate for the onset of puberty (Moschos *et al.*, 2002; Okusawa *et al.*, 2002; Zieba *et al.*, 2005). Recently it was further suggested that leptin would act in synergy with androgens to up-regulate the KiSS-system in humans (Morelli *et al.* 2008). Although evidence is scarce in teleosts, leptin was shown to modulate the release of gonadotropin hormones in

European sea bass, *Dicentrarchus labrax*, and rainbow trout according to their stage of gonadal development (Peyon *et al.* 2001; Weil *et al.* 2003). Furthermore, in the same species SL producing cells were sensitive to leptin only at the prepubertal and pubertal stage (Peyon *et al.*, 2003). The involvement of SL in the control of reproduction remains poorly characterized but its gene expression was up-regulated during vitellogenesis and spawning in Atlantic salmon broodstock (Benedet *et al.*, 2008). Finally, neuropeptide Y (NPY; regulating energy balance) and ghrelin (GH releasing-factor) are also likely to be involved in the metabolic regulation of sexual maturation in teleosts as in mammals. Both factors were indeed shown to interact with the BPG-axis but evidence remains limited as previously reviewed (Unniappan, 2010; Zohar *et al.*, 2010).

New mechanisms of photoperiod signal transduction to the BPG-axis are under investigation. Describing how these interact with nutritional factors is critical to the understanding of the plasticity of puberty onset in seasonal teleosts. The photoperiodic synchronization and developmental regulation of salmonid sexual maturation described in this review is likely to facilitate such undertakings by providing an hypothetical framework based on physiological, histological and life-history evidence.

## **6. The modulating role of temperature**

It must be acknowledged that photoperiod is not the only environmental factor influencing salmonid reproductive cycles. Other environmental cues include water temperature and salinity (Saunders *et al.*, 1994; Pankhurst and King, 2010) but social (e.g. pheromones) and behavioural (e.g. swimming exercise) factors could also be involved. These factors are recognized as secondary within the overarching reproductive strategy of salmonids but water temperature has a demonstrated modifying

role (Bromage *et al.*, 2001; Pankhurst and King, 2010; Taranger *et al.*, 2010). This is not surprising as ambient temperature can be expected to affect poikilotherm's metabolic and physiological processes related to both somatic and gonadal development. Within the normal thermal range of the species, temperature would act as a permissive factor of both male and female gametogenesis as shown by a higher rate of oocyte growth and plasma sex steroid level in males under increasing/higher water temperature (Pankhurst and King, 2010). However, water temperature above the range of thermal tolerance of the species compromises gamete quality (egg size, fertility, morphology and survival) through a thermal inhibition of vitellogenesis and  $17\beta$ -oestradiol secretion (King *et al.*, 2007; Pankhurst and King, 2010). The later stages of gonadogenesis (external vitellogenesis onward) were shown to be particularly sensitive to such thermal stress (King and Pankhurst, 2003). Closer to completion of maturation in autumn, salmonids delay or inhibit spawning when held under higher temperature (Gillet, 1991; Chmylevsky, 2000; King and Pankhurst, 2000; Taranger and Hansen, 2003) and inversely exhibit an earlier final maturation and ovulation under lower temperatures (up to 3-4 weeks advancement; Pankhurst and King, 2010). The effect of photoperiod shortening and temperature decrease are therefore considered additive with the latter acting as an ultimate factor fine-tuning the spawning-window to local thermal conditions so as to allow optimal timing for fry emergence (Taranger *et al.*, 2010). Overall, water temperature is an ultimate factor determining the optimal time for reproduction in salmonids (Taranger *et al.*, 2010). It is recognized secondary compared to photoperiod in the seasonal cueing, i.e. proximate control, of the reproductive cycle of salmonids (Bromage *et al.*, 2001; Pankhurst and King, 2010; Taranger *et al.*, 2010).

## 7. Conclusion

In Atlantic salmon, maturation was said to be “a cyclic process that begins at fertilization” and to be “continually repressed until the inhibitor is removed” (Thorpe *et al.*, 1998). Evidence reviewed here suggests that sexual development is not continually inhibited but seasonally inhibited under long-days only until the photo-inhibition is replaced by photo-stimulation if sufficient somatic resources and an acceptable level of gonadal development are reached. Thorpe *et al.* (1998) suggested that maturation will restart in November, i.e. at the onset of photo-neutrality, if the organism has sufficient energetic resources. It appears more appropriate to consider that sexual development will progress in correlation with somatic development under short-days (during the open-phase of the endogenous reproductive cycle) until the onset of differential photo-sensitivity.

Overall, the overarching reproductive strategy presented here is likely to facilitate the functional understanding of the PNES controlling the seasonal activity of BPG-axis in interaction with somatic factors but also the role of other ultimate factors such as water temperature and salinity. As in avian species, this could in turn facilitate the description of adaptative specialization and conditional plasticity in the photoperiodic response of the BPG-axis in relation to phylogenetic history between salmonid species and strains. More work is clearly needed to highlight fine-variations in the photoperiodic and developmental regulation of reproduction between salmonids. The correlation between body-growth and early gonadal development under short-days requires further assessment as this appears to be a critical factor for puberty onset and reproductive qualities such as fecundity and egg size. This could be responsible for the maturation at an earlier age and size of heavily exploited species as young would thrive on increased nutritional opportunities. However, strong reduction in homing rates

reported in salmonids could inversely, beside sea survival, be inherent to their diminishing growth and fitness. Post-smolt growth is indeed known to correlate with return rates (Friedland *et al.*, 1993; 2000) such that overexploitation of prey species (e.g. mesopelagic fishes and larger pelagic fish accounted for 66% and 13% by weight respectively of the stomach content of Atlantic salmon from the Northeast Atlantic; Jacobsen and Hansen, 2001) and/or cooling of sea surface temperature (such as observed in the North Sea; Friedland *et al.*, 2000) could affect return rate through their negative effect on maturation (Gende *et al.*, 2002; Hansen and Jacobsen, 2003). Overall, variations in sea surface temperature, as observed worldwide and in the North-East Atlantic ocean in particular, might increase post-smolt metabolic stress, modify migration pattern through behavioural thermoregulation and compromise the successful completion of reproduction through mechanisms requiring a better understanding of the annual reproductive cycle of salmonids.

The occurrence of a seasonal cycle of gonadal activity in immature and of aborted maturation in first-time spawners remains mainly hypothetical in salmonids and requires further assessment. The early growth history and somatic status of immature salmonids are likely to impact the maturation rate during subsequent breeding seasons. At an early stage, a feeding strategy limiting body fat accumulation under a decreasing or short-day photoperiod but favouring growth under long-days when sexual development is photo-inhibited is likely to prove beneficial in reducing subsequent pre-harvest sexual maturation at sea. Interestingly, an early transition from long-to-short days following the onset of photo-stimulation could interrupt sexual maturation in a proportion of fish that would otherwise complete maturation.

## **CHAPTER 2**

### **PAPER II**

#### ***REVIEW ARTICLE***

#### **REVIEW ON MORPHOLOGICAL SKIN COLOUR CHANGES IN TELEOSTS**

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**Abstract**

Morphological skin colour change in fish is often referred to in the sole context of background adaptation. It is becoming increasingly apparent that it is a broad phenomenon elicited from a variety of factors. To date, no review has attempted to integrate the different types of morphological colour changes occurring in teleosts, their ecological origins and the regulatory mechanisms involved, often restricting the view on the subject.

Firstly, the origin of skin colour is addressed in teleosts including chromatophore type and distribution, pigment biosynthetic pathways and their interactions to one-another. Secondly, the different types of morphological colour changes occurring in teleosts are categorized and a key distinction is made between proximate and ultimate morphological colour changes. These are defined respectively as the change of phenotype during an established life-stage in response to environmental interactions and during the transition between two developmental-stages phenotypically pre-adapted to their ancestral ecosystems. Nutrition and UV-light are primary factors of proximate morphological colour changes beyond the control of the organism. In contrast, background light conditions and social interactions are secondary proximate factors acting through the control of the organism. Highly diversified among teleosts, ultimate morphological skin colour changes are presented in terms of alterations in skin structure and pigment deposition during metamorphosis in different species. Finally, the physiological and endocrine mechanisms regulating both proximate and ultimate morphological colour changes are reviewed.

**1. Introduction**

The external colouration of fish is a distinctive feature which has long been of

interest to ecologists and biologists. It was recently shown that African cichlid (*Astatotilapia burtoni*, Cichlidae) can determine its social rank based solely on observation of interacting rivals (Grosenick *et al.*, 2007). Social interactions are strongly dependant on visual cues such as behaviour but also skin colour which is often used as a tool toward predator avoidance (camouflage, bartesian mimicry, palatability signal), prey capture (camouflage, aggressive mimicry), and conspecific communication (mating and agonistic signaling, shoaling preferences; Cheney *et al.*, 2008; Mills and Patterson, 2009). In ecological studies, it remains unclear how environmental and social signals regulate skin colour displacement and ultimately species divergence (Albert *et al.*, 2007). The formation of pigment pattern in model teleosts is a valuable system for studying developmental mechanisms and their evolution (Parichy, 2006). In cultured species, skin colour is observed daily as a general indicator of homeostasis, health and welfare but also of life-stage transition with practical implications. It is also an instinctive indicator of fish quality driving customer preference, hence a critical attribute to the ornamental and human food markets.

The external colouration of teleosts encompasses a variety of interests which reflects their own diversity and plasticity. The “eye-bar” of territorial male *Haplochromis burtoni* (Cichlidae) suddenly disappears when it is beaten by a rival and is soon followed by prompt retreat (Muske and Fernald, 1987). Within 2 to 8 seconds, tropical flounder *Bothus ocellatus* (Bothidae) achieve high-fidelity background pattern matching (Ramachandran *et al.*, 1996). Within a day on a white background, Australian snapper (*Pagrus auratus*, Sparidae) (Doolan *et al.*, 2009) becomes light coloured and commercially more valuable. Red sea bream (*Pagrus major*, Sparidae) reared under direct sunlight synthesizes more melanin, i.e. it ‘tans’, and loses its market value (Adachi *et al.*, 2005). In a few months, brownish Atlantic salmon (*Salmo salar*,

Salmonidae) parr become a shiny silvery smolt physiologically adapted to open-sea water environment. Female guppy (*Poecilia reticulata*, Poeciliidae) prefer males with more intense skin redness, driving through generations the development of such ornamental features (Grether *et al.*, 2005). Such examples of skin colour change in teleosts are recognized as occurring from two distinct phenomena, physiological and morphological colour changes. More comprehensively described, physiological colour changes are acute transient events caused by the motility of pigment vesicles or reflective structures within their cell (Bowman, 1942; Rodionov *et al.*, 1998, 2003). Primary physiological colour changes refer to the direct effect of environmental factors, such as light, on pigment migration (Oshima, 2001). Secondary physiological colour changes refer to the nervous and endocrine control of pigment translocation with a number of factors involved such as adrenocorticotrophic hormone (ACTH) and alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH) (Fujii, 2000). Nervous factors have a virtually instantaneous effect while the endocrine control is typically visible within minutes or hours (Fujii, 2000). In contrast, morphological colour changes are defined as occurring from variations in skin pigment concentrations and in the morphology, density and distribution of chromatophore in the three-dimensional organization of the integument (Chavin, 1969). Such colour changes are comparatively slow, occurring within days and weeks, with a more fundamental and long-lasting impact on external colouration. No classification is presently recognized with regards to describing this broad phenomenon. Critical for the survival, growth and propagation of teleost species, pigment displays are under strong evolutionary pressure. Following millions of years of natural and sexual selection, each teleost life-stage exhibits a phenotype which is not only pre-adapted to its ancestral habitat and strategy but also remains plastic for further adaptation to the actual environmental conditions. In this respect, we consider ultimate

morphological colour changes to occur during the *transition between two life-stages* phenotypically pre-adapted to their respective ancestral ecosystems, while proximate morphological colour changes occur during the *modulation of a life-stage phenotype* in response to on-going environmental interactions.

Based on this key distinction, this review aims at classifying, clarifying and unifying the different types of morphological colour change reported in teleosts, their ecological origins and physiological regulation to facilitate analysis in the various fields interested in teleost skin colour. This review is organized around three main topics. First, the origin of skin colour is described in fish including chromatophore types and distribution in the integument, pigment biosynthetic pathways and their interaction to one another. Secondly, the different types of morphological colour change witnessed in teleosts are reported and finally, for each of them, the physiological and endocrine factors identified are reviewed. Focusing on teleosts, reference is made to higher vertebrates and other species in which broader knowledge is available.

## **2. Origin of colour**

In teleosts, chromatophores originate from multipotent pigment cell precursors recruited from a pool of neural crest (NC) cells formed during embryogenesis. Chromatoblasts migrate along the dorsolateral and ventral-lateral pathways and differentiate into mature chromatophores which are ultimately distributed in the different regions of the integument where they give rise to the broad variety of colours and patterns observed (Sugimoto, 1993; Parichy, 2006).

### **2.1. Types of chromatophores and their organization within the integument**

#### **Chromatophore terminology**

Chromatophores, literally colour-bearers, are cells specialized in the storage and/or synthesis of light-absorbing (true) pigments or light-reflecting structures. Five main types of chromatophore are commonly distinguished, each presenting a specific colour range to the observer under white light: black-brown melanophores, red-orange erythrophores, ochre-yellow xanthophores, metallic iridescent iridophores and white/creamy leucophores.

Melanophores, erythrophores and xanthophores are dendritic cells specialized in the storage, but also the translocation, of their numerous light-absorbing pigment granules. They respectively store melanins, carotenoids and pteridines in their chromatosomes, namely melanosome, erythroosome and xanthosome, the latter being previously called pterinosome (Fujii, 1993; Fujii, 2000). Additionally, cyanophores are a newly recognized type of chromatophore responsible for vivid blue colouration found in some tropical fish species (Goda and Fujii, 1995). Data on cyanophores remains very scarce and they are not addressed further in this review. Iridophores are not dendritic cells and do not contain true pigment, instead they contain transparent purine crystal based reflecting-platelets (refractosomes). These organelles are stacked in a parallel fashion with alternating cytoplasmic layers of uniform thickness; a structural organization yielding iridescent properties and reflecting blue-green-metallic-colours according to the angle of observation (Menter *et al.*, 1979). The structural organization and reflected colours are not fixed. As described by Harris and Hunt (1973) in Atlantic salmon and by Hirata *et al.* (2003, 2005) in zebrafish (*Danio rerio*, Cyprinidae), the same species often exhibit different types of iridophores with regards to the size, number, orientation and distance between the reflecting platelets. Some teleosts also have the ability to alter the structural organization of their motile iridophores (Rohrlich, 1974; Fujii, 2000; Mäthger *et al.*, 2003). Leucophores are also light-reflecting cells but,

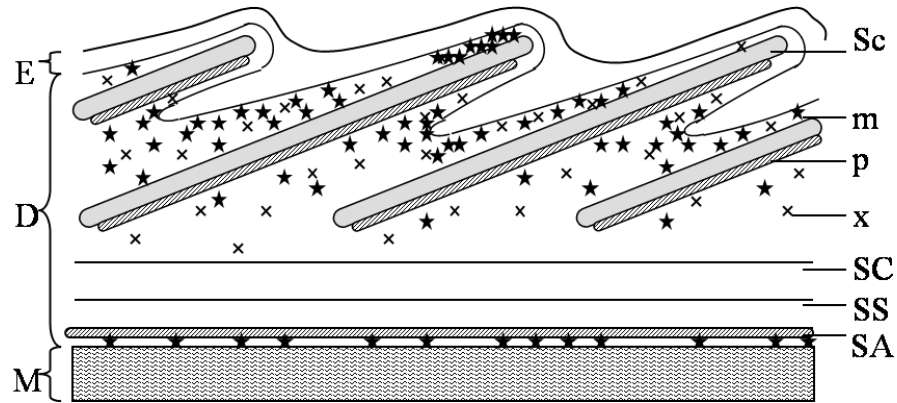
due to the poor organization of their purine crystals, they scatter light from a broad range of wavelengths producing an overall white/creamy colour. Their platelets are packed in leucosomes capable of migration in their dendritic cell such that, with regard to their morphological and motile characteristics, leucophores are more closely related to light-absorbing chromatophores. Furthermore, the distinction between leucophores and iridophores can be subjective with a range of intermediate structures described (Menter *et al.*, 1979).

### **Chromatophore distribution in the integument**

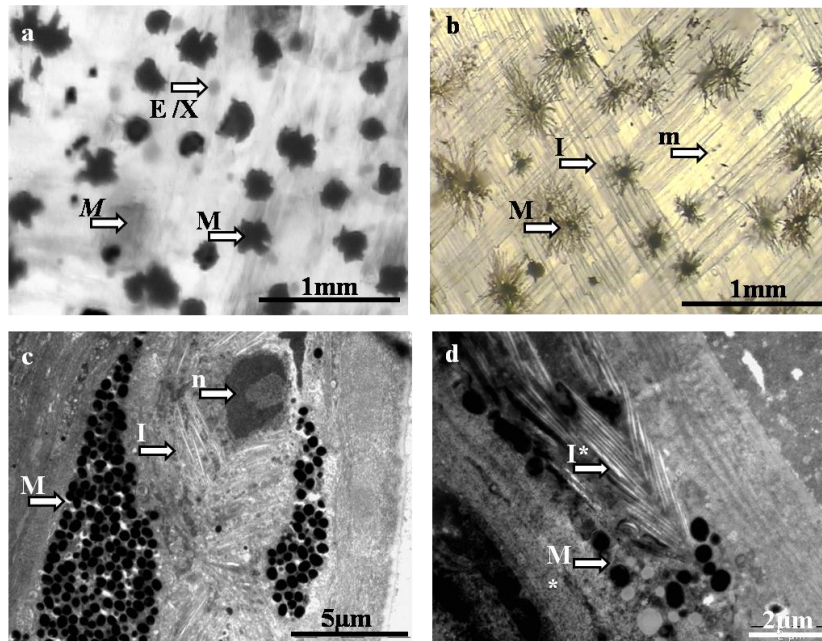
Observable colours are primarily dependent upon the morphology, density and distribution of the chromatophores within the three dimensional organization of the integument. Interactions between the different chromatophore layers allows the expression of various colours as recently illustrated in frogs and lizards by Grether *et al.* (2004) and in the striped pattern of the zebrafish by Hirata *et al.* (2003, 2005). Compared to amphibians, chromatophore distribution appears more complex within the scaled integument of teleosts where they are not necessarily organized in strict layers. A schematic representation (Fig.1.) along with macroscopic, microscopic and electron-microscopic pictures (Fig.2.) illustrates the distribution of chromatophores within the integument of Atlantic salmon.

Melanophores, mostly found in the dermis and sometimes the epidermis, are often associated with iridophores in melano-iridophore complexes. In this structural association, melanophores are located below the iridophores but extend their dendritic processes around them. When melanophores are in an aggregated state, only the light reflected by iridophores is visible as remaining wavelengths are absorbed by the underlying melanin. In a dispersed state, melanophores prevent light from reaching the

reflecting cells of the iridophores leading to skin darkening (Bagnara *et al.*, 1968; Fujii, 2000). This has been observed in,



**Figure 1.** Schematic diagram of Atlantic salmon dorsal integument (not to scale) (Modified from Hawkes, 1974; Johnston and Eales, 1967). **E:** Epidermis, **D:** Dermis, **M:** Muscle, **SS:** Stratum spongiosum, **SC:** Stratum compactum; **SA:** Stratum argenteum, **m:** Melanophore; **x:** Xanthophore, **p:** Purine crystal, **Sc:** Scale.



**Figure 2.** Photographs of **a)** pigment cells including melanophore (**M**), underlying melanophore (**M**) and erythro/xanthophore (**E/X**) on the dorsal skin area of an immature Atlantic salmon viewed from the external surface of the skin, **b)** pigment cells including melanophore (**M**), iridophore (**I**) and melanoblast (**m**) on the dorsal internal skin area of an immature Atlantic salmon viewed from the internal surface of the striped skin, **c)** and **d)** electron-photomicrographs of the dorsal area of an adult Atlantic salmon showing the melano-iridophore complex. **M\*:** melanosome, **I\*:** crystalline platelet, **n:** nucleus.

amongst other species, Atlantic salmon (Harris and Hunt, 1973), coho salmon (*Oncorhynchus kisutch*, Salmonidae) (Hawkes, 1974), turbot (*Scophthalmus maximus*, Scophthalmidae) (Huarong *et al.*, 2007), Mozambique tilapia (*Oreochromis mossambicus*, Percidae) (Lanzing and Bower, 1974) and in the dark facial stripe of nuptial male *Haplochromis burtoni* (Muske and Fernald, 1987). In amphibians, xanthophores strictly overlay the melano-iridophore complexes together constituting the dermal chromatophore unit providing pale colouring ranging from tan to green (Bagnara *et al.*, 1968). In salmonids, xanthophores and erythrophores are distributed below or above the clusters of iridophores and melanophores located in the dermis just beneath the epidermis. Lower in the dermis, pigments cells are concentrated below the *stratum compactum* on the lower boundary of the dermis where they constitute the *stratum argenteum* made of regularly packed iridophores with a few scattered melanophores (Fig.1.) (Johnston and Eales, 1967; Harris and Hunt, 1973; Hawkes, 1974). The *stratum argenteum*, also described in Mozambique tilapia (Lanzing and Bower, 1974) and mummichog (*Fundulus heteroclitus*, Fundulidae) (Menter *et al.*, 1979), is typically observed after filleting with silver white material left on the flesh and on the inside of the stripped skin. Particularly remarkable is the distribution of pigment-cells in the scale-less skin of the European eel (*Anguilla anguilla*, Anguillidae). Two chromatophore bilayers are found, one located at the interface of the epidermis and the dermis, and the other between the dermis and the subcutis lipid layer/muscle. Each bilayer is made of a pigment chromatophore layer superimposing a reflecting layer of light-reflecting chromatophores (Pankhurst and Lythgoe, 1982). Overall, a similar vertical organization of chromatophore strata was recently described in zebrafish with, from the surface, xanthophore/iridophore/melanophore/iridophore found in the dark stripe region and xanthophore/iridophore in the interstripe region (Hirata *et al.*, 2003,



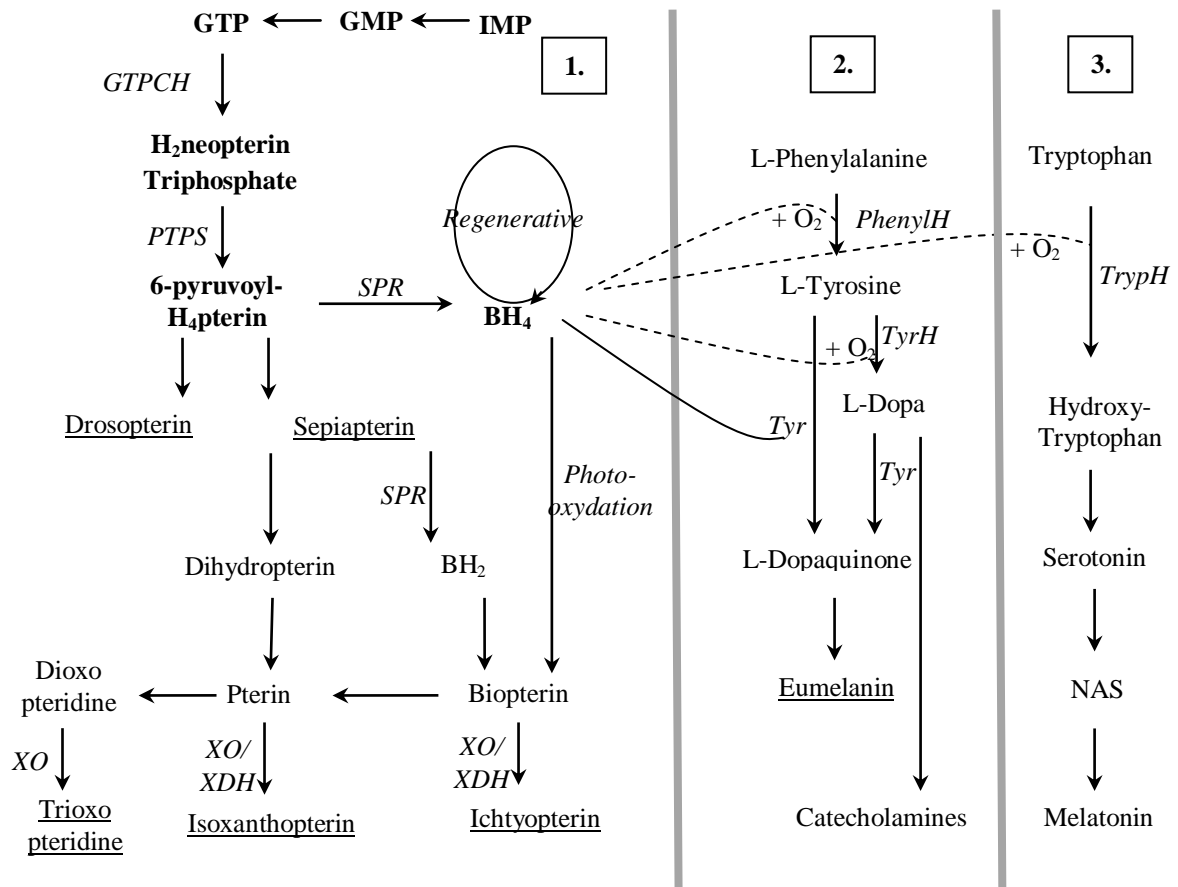
2005). Despite the variety of colours and patterns observed, the distribution of chromatophores in the integument appears to share some common structural organization in teleosts suggesting a common mechanism of development and colour revelation (Hirata *et al.*, 2003).

## 2.2. Pigment biochemistry and biosynthesis

### Melanins

Melanins are polymorphous and multifunctional biopolymers of high molecular weight and are among the most stable and insoluble biochemicals (Jacobson, 2000). Within the four groups of known melanins, allomelanin is restricted to fungi, plants and bacteria, neuromelanin to the central nervous system of some higher vertebrates (Fedorow *et al.*, 2005) and pheomelanin is not synthesized in fish melanophores, only eumelanin has been identified in teleosts (Bagnara and Matsumoto, 1998; Adachi *et al.*, 2005).

Eumelanin is primarily a light-absorbing pigment with an array of related ecological functions such as photoprotection, camouflage and communication. Additionally, other physiological functions are becoming increasingly evident with melanins acting as efficient antioxidants and immunostimulants, in accordance with their extra-cutaneous location (McGraw, 2005). The eumelanin biosynthetic pathway is highly conserved among vertebrates and occurs in melanosomes (Slominski *et al.*, 2004; Hoekstra, 2006). It was presented by Braasch *et al.* (2007) along with its enzymatic regulation by the tyrosinase (Tyr) gene family (Fig.3). Tyrosine hydroxylase (TyrH) activity and melanin synthesis has been detected in both ventral and dorsal skin of the African clawed frog (*Xenopus laevis*, Pipidae) (Zuasti *et al.*, 1998). Only recently was the tyrosinase gene family identified in Atlantic salmon skin (Thorsen *et al.*, 2006).



**Figure 3.** Pteridine synthesis pathway showing the central role of tetrahydrobiopterin (**BH4**) in melanogenesis and melatonin synthesis (Adapted from Braasch *et al.* 2007, Hoekstra *et al.* 2001; Wood *et al.* 1995; Schallreuter and Wood 1995; Ng 2009). **1.** The pteridine pathway is made of three inter-related compartments serving BH4 and pteridine pigment compounds synthesis: the *de novo* synthesis of BH4 (**Bold**), the regenerative pathway of oxidized BH4 (not described) and the biosynthesis pathway of yellow/red pteridine pigments (Braasch *et al.*, 2007). Coloured pteridines (underlined) and BH4 biosynthetic pathways have as a common substrate guanosine triphosphate (**GTP**), a purine nucleotide, synthesized from inosine monophosphate (**IMP**) through guanosine monophosphate (**GMP**). GTP is converted by GTP cyclohydrolase I (**GTPCH**) followed by 6-pyruvoyl tetrahydropterin synthase (**PTPS**) into 6-pyruvoyl tetrahydropterin, from where pigment pteridines and BH4 synthetic pathways diverge. The enzyme sepiapterin reductase (**SPR**) catalyses the last steps towards BH4 but is also involved in pteridine pigment synthesis together with xanthine oxydase (**XO**) and xanthine dehydrogenase (**XDH**) and other unknown factors. BH4 play a central role towards eumelanin and catecholamines synthesis as an essential co-factor **2.** by controlling the supply of L-tyrosine from L-phenylalanine by the enzyme phenylalanine hydroxylase (**PhenylH**) and L-dopaquinone from L-tyrosine by the enzyme tyrosine hydroxylase (**TyrH**). Furthermore, BH4 binds to the enzyme tyrosinase (**Tyr**) inhibiting its activity on the substrate L-tyrosine only. This inhibition can be relieved by the photo-oxidation of BH4 into dihydrobiopterin (**BH2**) and biopterin constituting a redox-switch controlling melanogenesis. Finally, **3.** BH4 is also an essential cofactor controlling the activity of the rate-limiting enzyme tryptophan hydroxylase (**trypH**) toward the synthesis of serotonin, N-acetyl-serotonin (**NAS**) and melatonin biosynthesis.

## Carotenoids

Carotenoids are lipid soluble organic pigments produced mainly in photosynthetic tissues. Around 600 carotenoids have been isolated from natural sources (Straub, 1987) of which up to 23 have been found in various fish tissues such as muscle, gonad, skin, liver, gut and kidney (Storebakken and No, 1992, Izquierdo *et al.*, 2005; Ytrestøyl and Bjerkeng, 2007). Astaxanthin (Ax) is the most abundant carotenoid in the aquatic ecosystem where it is naturally biosynthesized by phytoplankton while most crustaceans metabolize Ax from other carotenoids such as  $\beta$ -carotene, canthaxanthin (Cx) or zeaxanthin (Schiedt, 1998). As with other vertebrates, teleosts are unable to synthesize carotenoids *de novo* and rely solely on dietary supply to achieve their related red/orange to yellow skin pigmentation (Nickell and Springate, 2001).

Throughout the animal kingdom, carotenoids are often exhibited as an ornamental feature in nuptial displays. Their characteristic colours affect mate selection and reflect the foraging capacity, thus fitness of an organism (Grether *et al.*, 1999, 2005; Bourne *et al.*, 2003) particularly so since carotenoid compounds have an array of beneficial properties. They are efficient antioxidants protect cellular membranes against peroxide chain reaction of fatty acids (Palozza and Krinsky, 1992), free-radical scavengers or “super vitamin E” (Miki, 1991) and vitamin A precursors (Schiedt *et al.*, 1985) . Carotenoids also enhance the immune system (Christiansen *et al.*, 1995a) and Ax is associated with photoprotection against UV light through its free-radical scavenging properties (Sies and Stahl, 2004). Carotenoid absorption, metabolism and deposition in various organs varies according to dietary compound, species and tissue (Chatzifotis *et al.*, 2005). In farmed salmonids, synthetic Ax and to a lesser extent Cx are typical in-feed additives for flesh pigmentation (Storebakken and No, 1992; Bjerkeng, 2000). While 80% of the carotenoid compounds in the flesh are Ax, the skin

stores a more diversified range of metabolites dominated by salmoxanthin, zeaxanthin and various esters (Kitahara, 1985; Torrissen *et al.*, 1989).

### **Pteridines**

Pteridines are a family of heterocyclic compounds composed of pyrimidine and pyrazine rings and classified as pterins or flavins based on their structure. Most natural pteridines are easily oxidized, light-sensitive and poorly water-soluble. Types and abundance of pteridines in teleost skin are species, developmental stage and area specific. Xanthophores were first characterized in green swordtail (*Xiphophorus helleri*, Poeciliidae) with only some of their pteridine compounds being light-absorbing pigments (such as drosopterins, sepiapterin) with others being colourless but UV-fluorescent (such as xanthopterin, biopterin) (Matsumoto, 1965, Henze, 1977). Similarly in rainbow trout parr (*Onchorynchus mykiss*, Salmonidae), isoxanthopterin (IXP), ichtyopterin, biopterin and pterin were identified with the former two being the only coloured compounds (Premdas and Eales, 1976a and b). Most of the analyses performed to date to identify teleost's skin pteridines use thin-layer chromatography and UV-absorption spectrophotometry.

The biosynthesis pathway of pteridines in teleosts was reviewed by Ziegler (2003) and updated by Braasch *et al.* (2007) (Fig.3.). Sepiapterine-reductase (SPR) was only recently demonstrated in pigment cells of a teleost, the Japanese killifish (*Oryzias latipes*, Adrianichthyidae) (Negishi *et al.*, 2003). In the caudal fins of the guppy, 6-pyruvoyltetrahydropterin synthase (PTPS) activity was shown to be proportional to the density of xanthophores and erythrophores. In both those chromatophores, activity of Guanosine-5'-triphosphate (GTP) cyclohydrolase (GTPCH) and PTPS were significantly higher than in melanophores and non detectable in non-pigment cells (Ben

*et al.*, 2003).

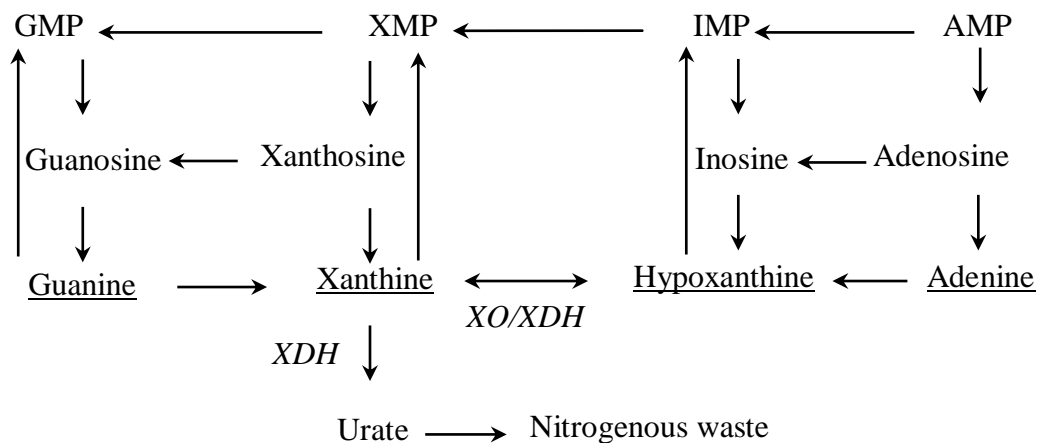
Drug-induced inhibition of xanthine dehydrogenase (XDH) significantly reduced the levels of xanthopterin (XP), IXP, biopterin and sepiapterin in the axolotl (*Ambystoma mexicanum*, Ambystomatidae) (Thorsteinsdottir and Frost, 1986). Overall, this clearly shows the pivotal role of those enzymes in pteridine pigment synthesis. With many pteridine compounds being colourless, they are likely to be involved in other intracellular or physiological processes. For example, 7-hydroxybiopterin was extracted from the skin of malabar danio (*Devario malabaricus*, Cyprinidae) and found to act as an alarm pheromone released upon skin injury and eliciting a fright reaction in conspecifics (Win, 2000). In mammals, activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to influence pteridine metabolism (Hoekstra and Fekkes, 2002; Hoekstra *et al.*, 2003). Neopterin was shown to be a mediator of the cellular immune system (Hoffman and Schobersberger, 2004). Finally, tetrahydrobiopterin (BH<sub>4</sub>) is a key pteridine compound best known as an essential cofactor for the synthesis of serotonin from tryptophan, and of tyrosine, dopamine and norepinephrine (NE) from phenylalanine, but has also less defined functions at the cellular level (Thöny *et al.*, 2000).

### ***Purines***

Purines are heterocyclic aromatic organic compounds made of a pyrimidine ring fused to an imidazole ring. They are biosynthesized as nucleotides, primarily in the form of inosine monophosphate (IMP), while free nucleobases (guanine (G), hypoxanthine (Hx), adenine and xanthine) are the primary product of purine nucleotide catabolism toward the excretion of nitrogenous waste (Fig.4.).

Guanine and hypoxanthine but also xanthine and to a lesser extent guanosine

monophosphate (GMP) and adenine are naturally occurring purine-based nucleobases constituting the main components of reflecting cells in vertebrates including teleosts (Johnston and Eales, 1967; Harris and Hunt, 1973; Premdas and Eales, 1976a; Oliphant and Hudon, 1993). Xanthine oxydase/xanthine dehydrogenase (XO/XDH) is the rate-limiting enzyme of purine catabolism catalyzing the oxidation of Hx into xanthine and xanthine into uric acid (Pritsos, 2000). In salmonids, purines isolated from rainbow trout parr and brook trout (*Salvelinus fontinalis*, Salmonidae) were mainly G and Hx with a reduced amount of GMP (Premdas and Eales, 1976a and b) whereas only G and Hx were identified in coho salmon (*Oncorhynchus kisutch*, Salmonidae) and Atlantic salmon (Markert and Vanstone, 1966; Johnston and Eales, 1967).



**Figure 4.** Schematic presentation of the purine catabolism pathway. Free purine bases can be reconverted to their corresponding nucleotide or further catabolised into urate (uric acid) towards the excretion of nitrogenous waste. Purine compounds stored as pigment in the skin are underlined. Xanthine oxydase (XO) and xanthine dehydrogenase (XDH) are critical for purine catabolism. **GMP**: guanosine monophosphate, **XMP**: xanthosine monophosphate, **IMP**: inosine monophosphate; **AMP**: adenosine monophosphate

### 2.3. Mosaic cells and biosynthetic pathway interactions

Mosaic pigment cells are occasionally observed in lower vertebrates and evidence on the coexistence/interactions between different pigment biosynthetic

pathways is increasing. Poorly referred to in the recent literature on teleost skin colour, this suggests that differentiated chromatophores could trans-differentiate, i.e. alter their pigment content hence their type, toward morphological colour change (Bagnara, 1983).

Erythrophores and xanthophores are well recognized as containing two kinds of pigment vesicles, erythroosome and xanthosome, the relative proportion of which distinguishes these two chromatophores although sometimes arbitrarily (Matsumoto, 1965). Both cells are capable of autonomous synthesis of pigment pteridines (Masada *et al.*, 1990). Mosaic pigment cells containing more than one type of organelle, and mosaic organelles containing more than one type of pigment can be found in vertebrate skin. In fact, in the leaf frog (*Phyllomedusa iheringii*, Hylidae) a mosaic chromatophore containing melanosomes, erythroosome, xanthosome and refractosome was observed (Bagnara, 1983). The author stated that “although each chromatophore type may be fully differentiated, their fates are not irrevocably fixed”. Occurrence of simultaneous cues for different pigment synthesis together with their common origin would allow chromatophore trans-differentiation and the occurrence of such mosaic cells (Bagnara *et al.*, 1979; Bagnara, 1983).

There is strong evidence supporting a likely cohabitation of both functional melanin and coloured pteridine pathways in melanophores and xanthophores. *In vitro*, amphibian xanthophores but also iridophores can transform into functional melanophores and vice versa (Bagnara *et al.*, 1978). In accordance, L-tyrosine (L-Tyr) was detected in goldfish (*Carassius auratus*, Cyprinidae) xanthosomes suggesting that erythrophores and xanthophores are in a labile state ready to undergo melanogenesis (Matsumoto and Obika, 1968). Conversely in Xiphophorine species, IXP, biopterin and sepiapterin were detected in melanophores (Henze, 1977) while goldfish melanophores similarly have a considerable capacity for pteridine biosynthesis, including the yellow

pigment sepiapterin (Masada *et al.*, 1990). More recently, a *de novo* BH4 biosynthetic pathway was shown to be involved in pteridine biosynthesis in guppy erythrophores and xanthophores and GTPCH and PTPS activity was detected in guppy melanophores although being higher in erythrophores (Ben *et al.*, 2003). This is, in fact, not surprising as it is well demonstrated that BH4, which shares common precursors with coloured pteridines, is an essential cofactor for Tyr activity (Fig.3.) in human melanocytes where BH4 *de novo* synthesis and recycling pathways occur (Schallreuter *et al.*, 1994). In addition, in human melanocytes, a rate limiting enzyme in BH4 regeneration pathway (pterin-4a-carbinolamine dehydratase, PCD) controls phenylalanine hydroxylase (PhenylH) enzyme activity and dissociates into a dimer (dimerisation cofactor of hepatocyte nuclear factor-1 (HNF-1), DcoH) which regulates the activity of the transcription factor (HNF-1) for phenylH but also Tyr gene expression (Schallreuter *et al.*, 2003). Together, this highlights the close proximity of melanin and pteridine pathways, suggesting that in teleosts established melanophores could differentiate into xanthophores and vice versa.

Oliphant and Hudon (1993) reviewed the presence of pteridines in iridophores and leucophores of various vertebrate species including amphibians and teleosts. They gathered evidence suggesting a possible role of pteridines in light reflecting pigment granules as, although being often colourless compounds, they can be found in crystal forms. In the yellow iris of the rock and ring dove (*Columbia livia* and *Streptopelia risoria*, Columbidae) "reflecting xanthophores" with the combined properties of both xanthophores and iridophores/leucophores were detected (Oliphant, 1987a and b; Tillotson and Oliphant, 1990). The transformation of purines into pteridines has already been clearly demonstrated *in vitro* (Albert, 1957) but *in vivo* evidence is still lacking despite long standing theories on the subject. Bagnara and Hadley (1973) stated that a



competition for substrates is likely to take place in organisms synthesizing both purines and pteridines. Indeed, in axolotls fed with an enriched guanosine diet, the density of both iridophore and xanthophore and their respective pigment contents significantly increased. This clearly indicates that both chromatophore types thrive on substrate from common origins while suggesting that pigment trans-differentiation might occur *in vivo* (Frost *et al.*, 1987). Overall, the interactions and common precursors leading to the synthesis of purine, pteridine and melanin pigments have been recently summarized based on the model zebrafish (Ziegler, 2003; Ng *et al.*, 2009). IMP is a common precursor for the synthesis of purine pigments, from AMP and GMP, but also, through GTP, of pteridine pigment and of BH<sub>4</sub> which plays a central role in eumelanin biosynthesis. In this respect, zebrafish mutants unable to synthesize IMP or zebrafish where GMP synthesis was disrupted exhibit almost no xanthophores or iridophores and a significantly decreased melanin concentration (Ng *et al.*, 2009). Variations in intracellular levels of GTP and GMP could alter pathway prevalence for pteridine, purine and melanin (through BH<sub>4</sub>) pigment biosynthesis.

The interactions and expression of different pigment biosynthetic pathways within an established chromatophore could allow its trans-differentiation through variations in enzyme activities or altered availability of intracellular precursors. Remaining mainly speculative *in vivo*, this interesting phenomenon requires further consideration and could benefit from recent advances in the knowledge of the mechanisms of chromatophore differentiation and pattern development.

### **3. Types of morphological skin colour changes**

Defined as variations in skin pigment concentrations and/or alterations in the morphology, density and distribution of chromatophore in the three-dimensional

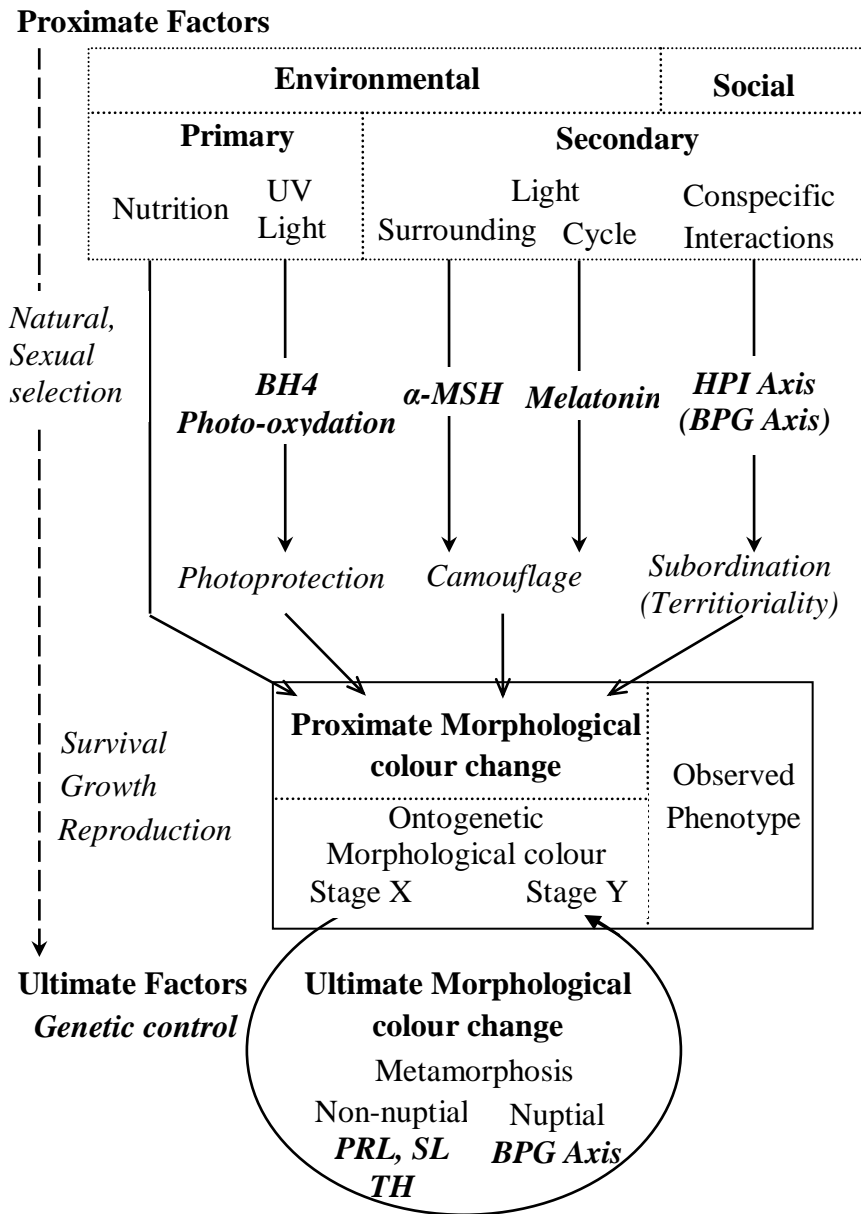
organization of the integument (Chavin, 1969), it is becoming increasingly apparent that skin morphological colour change is a broad phenomenon elicited from a variety of factors which have not been co-jointly addressed and categorized.

### 3.1. Terminology

Kodric-Brown (1998) defined the expression of nuptial chromatic traits in fish as arising from proximate mechanisms and ultimate evolutionary selective processes. In this respect, for the purpose of this review and future work, we propose to distinguish ultimate and proximate morphological colour changes from an ecological perspective. Over millions of years of evolution, the genetic basis providing an organism with the fittest phenotype has been selected. This includes both a pigment pattern adapted to the ancestral habitat and behaviour of the species developmental-stage but also the ability to modulate it in the presence of proximate environmental variations, both phenomena together allowing optimal adaptation.

The observed skin colour is an innate characteristic with a degree of plasticity elicited from environmental interactions. In this sense, ultimate morphological colour changes are defined as occurring during the *transition between two life-stages phenotypically adapted to their ancestral ecosystems* such as the larvae/juvenile, juvenile/adult, immature/nuptial metamorphosis. They are often concomitant with niche-shift and related alterations e.g. feeding habit, prey/predator relationship, abiotic characteristic of the environment and/or with a new life-stage strategy e.g. growth to reproduction. Often dramatically altered to preserve fitness within the new ecosystem, the newly exhibited phenotype is essentially an intrinsic characteristic of the species next developmental-stage. This can also share common features within a group of species either phylogenetically related (sharing homologous external colouration from a

common ancestor) or evolutionarily convergent (sharing analogous external colouration arising from common selective pressure). In contrast, proximate morphological colour changes are defined as the *morphological modulations of a given life-stage skin colour in response to occurring variations in biotic and abiotic environmental factors*. This adaptative mechanism allows fine-tuning of the ultimate morphological colour of an organism developmental-stage in the presence of varying environmental conditions in its ecological niche. For instance in salmonids, the parr/smolt and immature/nuptial adult transformations are characteristic ultimate morphological colour changes concomitant with diadromous migration, and share strong resemblance between species of this family regardless of individual environments. In addition, each of these life-stages could undergo proximate morphological colour changes in response to occurring alteration e.g. nutrition and surrounding light conditions. Furthermore, as addressed by Fuji (2000), an environmental factor is said to be primary or inducing a primary colour response when it has a direct effect on chromatophores beyond the control of the organism. In contrast, a secondary colour response is under direct neuronal or endocrine control from the organism, hence elicited from environmental factors which have indirect effects on the chromatophore. According to these definitions, the different types of morphological colour changes reported in this review are presented schematically (Fig.5.). The main teleosts species in which these specific morphological colour changes were observed are further listed in Table 1 and 2.



**Figure 5.** Schematic representation of the different types of morphological colour changes, their origin and main factors.  **$\alpha$ -MSH**: Alpha-melanocyte-stimulating hormone; **BH4**: Tetrahydrobiopterin; **BPG**: Brain- pituitary-gonadal; **HPI**: Hypothalamic-pituitary-interrenal; **PRL**: Prolactin, **SL**: Somatolactin, **TH**: Thyroid hormone.

**Table 1.** Different types of proximate morphological colour changes observed in teleosts with type of chromatophore affected.

Type	Chromatophore	Species	References	
<b>Nutrition</b>	M	Flatfish juvenile	Venizelos and Benetti, 1999 Bolker and Hill, 2000 McEvoy <i>et al.</i> , 1998 Estévez <i>et al.</i> , 1999, 2001	
		E/X	Chinook salmon fry Trout Atlantic cod Red porgy Red sea bream Australian snapper Channel catfish Banded cichlid Goldfish, koi carp Blue tilapia	Nikolaides and Woodall, 1962 Peterson <i>et al.</i> , 1966 Gosse and Wroblewski, 2004 Chatzifotis <i>et al.</i> , 2005 Izquierdo <i>et al.</i> , 2005 Ibrahim <i>et al.</i> , 1984 Booth <i>et al.</i> , 2004 Li <i>et al.</i> , 2007 Kop and Durmaz, 2007 Gouveia <i>et al.</i> , 2003 Shiau and Lin, 2006
	<b>Photoprotection</b> <i>UV-B induced skin darkening</i>  <i>UV-B induced skin damage</i>	M	Hammerhead shark	Lowe and Goodman-Lowe, 1996
			Whitefish larvae	Häkkinen <i>et al.</i> , 2002
			Red sea bream	Adachi <i>et al.</i> , 2005
			Trouts	Fabacher and Little, 1996
			Atlantic salmon	McArdle and Bullock, 1987
			Cutthroat trout	Blazer <i>et al.</i> , 1997
			Sole, turbot larvae	McFadzen <i>et al.</i> , 2000
			Eurasian minnow	Kaweewat and Hofer, 1997
			Rainbow trout	
			Arctic charr	
	<b>Cryptism</b> <i>Surrounding light</i>	M	<i>Lebistes, Erricynba, Fundulus, Ameirus, Macropodus, Gambusia, Atherina, Paralichthys</i>	Sugimoto, 2002
Japanese killifish			Fukamachi <i>et al.</i> , 2004	
Flatfish			Venizelos and Benetti, 1999 Bolker and Hill, 2000	
Mozambique tilapia			van Eys and Peters, 1981	
E/X M,I,X M, I/L		Brook trout fry	Donnelly and Whoriskey, 1991	
		Brook trout	Peterson <i>et al.</i> , 1966	
		Zebrafish	Sugimoto <i>et al.</i> , 2005	
		Opaleye	Sumner, 1943, 1944	
<b>Communication</b> <i>Subordination</i>  <i>Territoriality</i>	Darkening	A. salmon juvenile	O'Connor <i>et al.</i> , 1999, 2000	
		Arctic charr	Höglund <i>et al.</i> , 2000	
		Mozambique tilapia	Lanzing and Bower, 1974	

**M:** Melanophore, **E:** Erythrophore, **X:** Xanthophore, **L:** Leucophore, **I:** Iridophore.

**Table 2.** Different types of ultimate morphological colour changes observed in teleosts with type of chromatophore affected.

Type	Chromatophore	Species	Reference
<b>Non-Nuptial Metamorphosis</b>			
	M	Flatfish Larvae/Juvenile	Venizelos and Benetti, 1999 Bolker and Hill, 2000 Hamre <i>et al.</i> , 2007
	X	<i>Xiphophorus</i> sp.	Matsumoto, 1965 Henze <i>et al.</i> , 1977
	M/X/E	Zebrafish	Parichy, 2006 Hirata <i>et al.</i> , 2003, 2005
	E	Salmonid parr/smolt	Storebakken <i>et al.</i> , 1987 Bjerkeng <i>et al.</i> , 1992
	I/L	Salmonid parr/smolt	Johnston and Eales, 1967 Vanstone and Market, 1968 Haner <i>et al.</i> , 1995
<b>Nuptial Metamorphosis</b>			
	E/X	Salmonids	Kitahara, 1983, 1985 Bjerkeng <i>et al.</i> , 1992 Salzburger <i>et al.</i> , 2007
		Cichlids	
		Teleosts	Darwin, 1890, Kodric-Brown, 1998
		Tilapia	Lanzing and Bower, 1974
		Fathead minnow	Pawlowski <i>et al.</i> , 2004
	I/L	Eels	Pankhurst and Lythgoe, 1982

**M:** Melanophore, **E:** Erythrophore, **X:** Xanthophore, **L:** Leucophore, **I:** Iridophore.

### 3.2. Proximate morphological colour changes

Four environmental factors eliciting different types of proximate morphological colour changes in teleosts are reported and hereby reviewed: Nutrition and colour expression, solar-radiation and photoprotection, surrounding luminosity and background adaptation and finally social interactions and visual communication mainly through the melanogenic effect of stress. Nutrient availability and UV are primary factors, while background reflectance and social interactions are secondary factors of proximate morphological colour change.

### **Nutrient availability and colour expression**

The impact of nutrition on skin pigmentation in teleosts is well documented. It has mainly been studied in aquaculture species in which skin carotenoid content is a valuable trait, but also in flatfish where a high incidence of albinism can occur under artificial rearing conditions.

In many species visibly accumulating carotenoid compounds in the integument, expression of the wild-type phenotype can be impaired when they are fed an artificial diet. In immature red porgy (*Pagrus pagrus*, Sparidae), individuals fed carotenoid-free diet have no detectable level of this pigment in the integument. Significant improvements arose from tailoring the carotenoid-feeding strategy in terms of content, composition and duration of the regime with esterified Ax giving the best results (Chatzifotis *et al.*, 2005; Izquierdo *et al.*, 2005). Similarly in Australian snapper (*Pagrus auratus*, Sparidae) skin redness decreased progressively when fish were fed an Ax free diet and was significantly improved when fish were fed a higher Ax concentration (Booth *et al.*, 2004). In wild Atlantic cod (*Gadus morhua*, Gadidae), the golden/reddish-brown colour of the skin originates from an invertebrate based diet rich in carotenoids which was also lost when reared under artificial conditions (Gosse and Wroblewski, 2004). The effect of dietary carotenoids on the expression of skin colour has also been reported in red sea bream (Ibrahim *et al.*, 1984), channel catfish (*Ictalurus punctatus*, Ictaluridae) (Li *et al.*, 2007), banded cichlid (*Cichlasoma severum*, Cichlidae) (Kop and Durmaz, 2007), goldfish and koi carp (*Cyprinus carpio*, Cyprinidae) (Gouveia *et al.*, 2003). At the breeding stage, expression of the carotenoid based nuptial colouration of guppy is limited by carotenoid availability in the wild (Grether *et al.*, 1999). In salmonids, efforts toward tailoring carotenoid-supplementation regime focus on achieving desirable flesh redness intensity (Bjerkeng, 2000). From hatching to harvest,

no report exists on variations in skin pigmentation with the carotenoid content of the artificial diet. Brook, brown and rainbow trout were shown to deposit an undesirable yellow pigment in the skin when fed a paprika pepper extract and a yellow golden colour when lutein was incorporated in the diet (Peterson *et al.*, 1966).

In farmed flatfish, nutritional deficiencies were shown to impair melanogenesis leading to skin pigmentation abnormalities. Occurrence of partial or total albinism varied with formulation of the larval diet (species and origin of the live preys, their enrichment and inter-conversion). Critical dietary requirements are fat soluble vitamins and lipids (essential fatty acids, vitamin A and D) but also carotenoids and riboflavin (reviewed by Venizelos and Benetti, 1999; Bolker and Hill, 2000; Hamre *et al.*, 2005). Experimentally, a high incidence of malpigmentation is observed in flatfish fed live prey enriched with arachidonic acid (ARA) prior to metamorphosis (McEvoy, 1998; Estévez *et al.*, 1999, 2001). Evidence of such nutritional impacts on melanogenesis are not restricted to Pleuronectiformes. Chinook salmon fry (*Oncorhynchus tshawytscha*, Salmonidae) show marked depigmentation possibly through impaired melanization when fed an essential fatty acid/fat free diet (Nicolaides and Woodall 1962). In red porgy, besides the carotenoid requirement, skin melanin was significantly increased probably from the high protein/carbohydrate ratio of the artificial diet (Chatzifotis *et al.*, 2005). Finally, other nutritional deficiencies can also lead to colour alterations such as sub-optimal vitamin-E in blue tilapia (*Oreochromis aureus*, Cichlidae) (Shiau and Lin, 2006). In humans, zinc deficiency was also shown to impair normal melanogenesis (Brüske and Salfeld, 1987).

While no such reports exist for pteridine and purine pigments, various studies have demonstrated a dietary effect on carotenoid and melanin accumulation with potential impact on both reared and wild-stock market values. Improper feed



composition, unforeseen variations in diet quality or poor feeding alone can lead to malpigmentation under domestication. Under natural conditions, a given species evolving in distinct habitats and feeding on different diets can also exhibit variations in skin colouration, as shown in wild cod (Gosse and Wroblewski, 2004). In ecological studies, nutrition is a basic criterion to consider when analysing skin colour displacement and species divergence in wild populations inhabiting different habitats. In a behavioural study, it was shown that firemouth cichlid males (*Cichlasoma meeki*, Cichlidae) fed a high carotenoid diet won a higher proportion of contests against conspecifics fed a low carotenoid diet (Evans and Norris, 1996). Colour expression alone can alter social status and dietary alterations could also have profound consequences on organism homeostasis through impairment of melanogenesis. Melanin is indeed the prime pigment recruited for photoprotection, camouflage and visual communication. These adaptative mechanisms of proximate morphology are critical and described hereafter.

### **UV-induced sun tanning: photoprotection**

As previously reviewed in fish, UV radiation has damaging properties on many biological molecules such as DNA and proteins (Zagarese and Williamson, 2001). They can cause upon excessive exposure eye and skin damage followed by secondary infections (Sharma *et al.*, 2005), increased mortality particularly of early life-stages (Bell and Hoar, 1950; Hunter *et al.*, 1981; Walters and Ward, 1998), immunosuppression (Salo *et al.*, 1998; 2000a; Jokinen *et al.*, 2001), stress response (Salo *et al.*, 2000b; Alemanni *et al.*, 2003) and shade-seeking behaviour with a high selective avoidance of UV radiation ( $\lambda$ 280–400 nm) (Kelly and Bothwell, 2002).

UV radiation induced alteration in the superficial skin structure (reduced density of epidermal goblet and mucous cells, alteration in mucous production, epidermis sloughing) in, amongst other species, razorback sucker (*Xyrauchen texanus*, Catostomidae) (Blazer *et al.*, 1997), common minnow (*Phoxinus phoxinus*, Cyprinidae), Danube bleak (*Chalcalburnus chalcoides*, Cyprinidae) rainbow trout and Arctic charr (*Salvelinus alpinus*, Salmonidae) (Kaweewat and Hofer, 1997), sole (*Solea solea*, Soleidae) and turbot larvae (McFadzen *et al.*, 2000). More extensive skin damage (skin lesion, epidermis hyperplasia and necrosis) was also reported in Lahontan cutthroat trout (Blazer *et al.*, 1997) and in sea-reared Atlantic salmon where it was called UV induced “summer syndrome” and induced increased stock mortality (McArdle and Bullock, 1987).

By absorbing wavelengths throughout the UV and visible spectrum, melanin acts as an optical screen attenuating the penetration of UV into the underlying tissue (Kollias *et al.*, 1991). The first case of UV-induced morphological colour change was reported in a cartilaginous fish, scalloped hammerhead shark (*Sphyrna lewini*, Sphyrnidae) kept in shallow water (Lowe & Goodman-Lowe, 1996). Later, whitefish larvae (*Coregonus lavaretus*, and *Coregonus albula*, Salmonidae) were shown to increase skin melanin concentrations when exposed to artificially high UV-B radiation from 2 weeks post-hatching (Häkkinen *et al.*, 2002). Adult red sea bream reared without shading-cover displayed a sharp decrease in skin luminosity, a 5-fold increase in eumelanin precursor and an increased density of enlarged melanocytes constituting a continuous monolayer at the surface of the skin when compared to shaded and wild individuals (Adachi *et al.*, 2005). Similarly, 48h of exposure to UV-B radiations induced skin darkening through melanophore dispersion (physiological colour change)

in rainbow trout and Lahontan cutthroat trout (*Oncorhynchus clarki henshatvi*, Salmonidae) (Fabacher and Little, 1996).

Despite the widespread damaging effect of UV-B in teleosts, documented cases of UV-induced melanogenesis for photoprotection remain limited in teleosts. This is probably because skin melanin content is rarely quantified when studying UV-induced skin damage. “Suntanning” in fish is likely to be more common than expected but also likely to vary with species, duration and intensity of exposure making experimental assessment difficult.

### **Background adaptation: camouflage**

Based mainly on work with Japanese killifish, it became clear in the early 20<sup>th</sup> century that long-term adaptation to a black or white background induced, following melanosome migration, variations in melanophore size, density (multiplication or degeneration) and skin melanin content (Odiorne, 1933, 1948). Confirmed more recently (Sugimoto, 1993), it is now recognized that the major cue for background adaptation or camouflage is in fact “the ratio of the intensities of light from above and from below “albedo”” (Sugimoto, 2002). The penetration of light in the aquatic ecosystem together with the substrate reflectance is therefore an important factor which varies with water spectral absorbance and turbidity but also with recurrent climate pattern and latitude. Background adaptation is the most comprehensively studied mechanism of proximate morphological colour change and is a recurrent phenomenon in teleost species as reviewed by Sugimoto (2002). Mozambique tilapia exposed to a black background increased the density of their dermal melanophore within 14 days (van Eys and Peters, 1981). After 12 weeks of acclimation in tanks matching the background colours of the arena where a predator challenge was performed, brook trout

fry underwent cryptic morphological colour change and their predation by birds was significantly reduced compared to non acclimated fish (Donnelly and Whoriskey, 1991). The effect of background adaptation on other chromatophores is much less studied. Early work with the opaleye (*Girella nigricans*, Kyphosidae) showed that skin guanine (G) concentrations significantly increased with an increasing level of background albedo while melanin concentration decreased (Sumner, 1944).

Exposed to a light background, brook trout mobilize their carotenoid pigment from the skin to the flesh, a process reversed following return to their original dark background (Peterson *et al.*, 1966). The dorsal skin of European eel maintained in total darkness showed a reduction in purine concentrations (Pankhurt and Lythgoe, 1982). In Japanese killifish, leucophore density increased while melanophore and xanthophore density decreased upon acclimation to a white background while the opposite was observed on a black background (Sugimoto *et al.* 2000; Fukamachi *et al.* 2004). More recently, it was shown in zebrafish that superficial melanophore and iridophore density and distribution varied within a week allowing adaptation to a white or black background. When exposed to a white background for several months, hypodermal chromatophores were also affected with a decrease in melanophore and an increase in xanthophore densities (Sugimoto *et al.*, 2005). Together, all these findings constitute strong evidence that the density of each chromatophore type can be altered to achieve optimal camouflage.

### **Social interactions and intra-specific communication**

Many teleosts exhibit an intrinsic phenotype at a given developmental-stage strategically affecting their social interactions as a result of selective pressure. Cases of social interactions inducing proximate morphological colour changes remain limited to

date in the literature. Morphological colour changes found between juvenile and adult emperor angelfish, *Pomacanthus imperator* (Pomacanthidae) as an adaptation against intraspecific antagonistic interactions should be considered as ultimate and do not apply in this section (Fricke, 1980; Bellwood *et al.*, 2004). Recently, it was shown that bluestriped fangblenny (*Plagiotremus rhinorhynchus*, Blenniidae) changes its colour to mimic its model, a juvenile cleaner fish (*Labroides dimidiatus*, Labridae) within 30min of cohabitation (Cheney *et al.*, 2008). This first documented case of facultative mimicry arose in light of data from physiological colour change. In *Pundamilia nyererei* (Cichlidae) as in other cichlids, male-male interaction leads to increased nuptial-colouration (Dijkstra *et al.*, 2007). Male *Haplochromis burtoni* switching from being non-territorial to territorial develop brighter colours while the opposite is observed when returning to a non-territorial state (Francis *et al.*, 1993; Korzan *et al.*, 2008). In bluehead wrasse (*Thalassoma bifasciatum*, Labridae), larger females can become dominant in the absence of dominant males and undergo behavioural and morphological sex change while developing the dominant male phenotype (Semsar and Godwing, 2003). Similarly in Mozambique tilapia, an extensive study of colour pattern in relation to behaviour demonstrated that colour was significantly altered in territorial males (Lanzing and Bower, 1974). Furthermore, eye colour was shown to indicate social rank in juvenile Atlantic salmon (Suter and Huntingford, 2002) and Nile tilapia (*Oreochromis niloticus*, Cichlidae) (Volpato *et al.*, 2003). Interestingly, it was shown that social subordination following aggression resulted in significant body darkening (submissive body darkening) in juvenile Atlantic salmon and Arctic charr (O'Connor *et al.*, 1999, 2000; Höglund *et al.*, 2000). In these experiments chromatophore density or pigment concentration were not measured and physiological colour change was likely to be involved. Nonetheless in some of these studies body darkening was shown to

originate from stress related endocrine factors known to induce morphological colour change as will be described later.

### **3.3. Ultimate morphological colour changes**

Ultimate morphological colour changes in teleosts species cannot be exhaustively enumerated and a good example was provided by Parichy (2006) describing differences in melanophore, xanthophore and iridophore distribution between the larval and adult stage in the model species zebrafish. In flatfish, these are particularly dramatic during larvae/juvenile metamorphosis, have been extensively reviewed and will not be addressed further (Venizelos and Benetti, 1999; Bolker and Hill, 2000). Similarly, chromatophore distribution and related skin pattern can be drastically altered and readily observable in various tropical/coral teleosts during metamorphosis (Fricke, 1980; Bellwood *et al.*, 2004).

This section will focus on nuptial metamorphosis and transition to the adult stage in diadromous species where data are more extensively available. Following an overview of skin colour and structure alterations during sexual metamorphosis, different examples illustrating variations in pigment content and chromatophore density during life-stage transition in teleosts are provided.

#### **Colour changes and skin structure alteration during sexual metamorphosis**

As listed by Darwin (1890) and more recently by Kodric-Brown (1998), sexual maturation is often concomitant with the development of nuptial colouration, which can also be sexually dimorphic, in an extensive range of fish species. A few examples are European eel, American eel (*Anguilla rostrata*, Anguillidae) and Japanese eel (*Anguilla japonica*, Anguillidae) which have a silvery-white belly and a dark back when sexually

mature compared to the yellowish-white belly and dorsal green-brown shades when immature (Pankhurst and Lythgoe, 1982). In these species, “silvering” of the skin is a reliable criterion of gonadal development (Han *et al.*, 2003). Nuptial metamorphosis is particularly pronounced in salmonids with the development of a distinctive brown-red colouration with added red marks in males compared to the characteristic silver cover of immature adults. Mozambique tilapia was shown to exhibit distinctive colour patterns at the juvenile, adult, female courting and female brooding and territorial male stages partly due to morphological colour changes (Lanzing and Bower, 1974). Mature male haplochromine cichlids develops conspicuous yellow to orange “anal ocelli” or egg-dummies near the base of the anal fin to attract females and optimize egg fertilization (Wickler, 1962, Salzburger *et al.*, 2007). Mature male fathead minnow (*Pimephales promelas*, Cyprinidae) have a darker body colour (Pawlowski *et al.*, 2004).

Thickening of the epidermis and dermis of either or both sexes also occurs during the reproductive cycle of many teleost species. It is a well recognized secondary sexual characteristic in, among others, three-spined stickleback (*Gasterosteus aculeatus*, Gasterosteidae) (Burton, 1979), winter flounder (*Pseudopleuronectes americanus*, Pleuronectidae) (Burton and Burton, 1989), European perch (*Perca fluviatilis*, Percidae) (Lindesjö, 1994), European eel (Saglio *et al.*, 1988), rainbow trout (Nakari *et al.*, 1986), brown trout (Pickering, 1977; Pottinger and Pickering, 1985), sockeye salmon (*Oncorhynchus nerka*, Salmonidae) (Johnson *et al.*, 2006), adult and precociously mature parr Atlantic salmon (Wilkins and Jancsar, 1979; Burton *et al.*, 1985). Scale resorption and variations in epidermal secretary cell populations (mucous cells and club cells) also occur during the spawning season in several species (Pickering, 1977; Nakari *et al.*, 1986; Northcott and Bullock, 1991; Irving, 1996; Kacem and Meunier, 2003). Such structural alterations of the skin are likely to play a significant role in ultimate

morphological colour change by altering the distribution of chromatophores. Maturing Atlantic salmon undergo both skin thickening and scale resorption such that purine crystals located immediately below the scale surface are both reduced in quantity but also move deeper into the dermis and are hence less visible. This would allow erythrophores, besides their higher density and pigment content, to appear more superficially located. Finally, the structural association of melanophores and erythrophores is also clearly visible in nuptial males where a high density of erythrophores covers the melanophore black-marks from the outside inward resulting in a red-ring and eventually a plain red-mark (Eric Leclercq, personal observation).

### **Alteration in pigment deposition**

#### *Carotenoids*

Carotenoid dynamics throughout the salmonid life-cycle have been extensively studied as a phenomenon of ecological interest with commercial implications in relation to whole fish quality. Carotenoids are found in a number of organs including the liver for metabolization and excretion but vary mainly between three compartments: flesh, skin and gonads (Hardy *et al.*, 1990; Storebakken and No, 1992; Ytrestøyl and Bjerkgeng, 2007) with flesh said to act as a “sink” in the sea and a “source” during freshwater homing (Rajasingh *et al.*, 2006).

In juveniles, a large proportion of the total body carotenoid is found in the skin and none in the flesh. Later in life, and concurrent with the completion of smoltification, carotenoid concentration increases steadily in the flesh, while decreasing in the skin despite the use of carotenoid rich diets under culture conditions (Storebakken *et al.*, 1987, Torrissen *et al.*, 1989, Bjerkgeng *et al.*, 1992). In immature adults of both sexes, more than 90% of the total body carotenoids are found in the flesh (Kitahara, 1983,



Bjerkeng *et al.*, 2000). Sexually mature female Atlantic salmon retain on average about 40% of their initial flesh carotenoid levels in this compartment (Torrissen and Torrissen, 1985). Flesh carotenoid loss is primarily concomitant with a total body carotenoid loss such that, in rainbow trout, sexually mature females and males retained 79% and 18% respectively of their initial total body carotenoids (Bjerkeng *et al.*, 1992). Carotenoid pigments are also redistributed from the flesh to both the skin and gonad. Up to 18% of the total body carotenoids may be present in ripening trout eggs (Sivtseva, 1982) and carotenoids were also reported in testis of rainbow trout, common whitefish (Czeczuga, 1975) and Atlantic salmon (Leclercq, unpublished data). In the skin, carotenoid levels increase as maturation progresses to reach about 8 times the pre-spawning levels in chum salmon (*Oncorhynchus keta*, Salmonidae) (Kitahara, 1983), 2.4 times in masu salmon (*Oncorhynchus masou*, Salmonidae) (Kitahara, 1985), 2.6 and 1.4 in male and female rainbow trout respectively with varying profiles (Bjerkeng *et al.*, 1992). Carotenoids are much less studied in other species but are clearly deposited in the skin during the breeding season in many teleosts such as guppy (Grether *et al.*, 1999), two-spotted gobies (*Gobiusculus flavescens*, Gobiidae) (Svensson *et al.*, 2007), three-spined sticklebacks (Barber *et al.*, 2000) and the haplochromis cichlid *Pundamilia nyererei* (Dijkstra *et al.*, 2007).

### *Purines*

Characteristic of salmonid species, the parr-smolt transition is one of the most significant colour changes with respect to purine deposition and was first studied by Johnston and Eales (1967). In the parr stage, the typical two layers of iridophore within the skin structure are thin but present. The inner (“skin”) layer underwent a sharp increase in purine content in January and February, corresponding to the onset of

smoltification and an increasing silvery appearance of the parr, followed by a steady deposition until June. The inner layer always maintained a higher content than the outer (“scale”) layer which accumulated purine compounds from March to May, corresponding to the completion of smoltification. Similarly in other smolting salmonids, iridophore density increased along with purine concentration in coho salmon (Vanstone and Market, 1968) while skin reflectance and silvering positively correlated with skin G concentration in steelhead rainbow trout and chinook salmon (Haner *et al.*, 1995). Nuptial Atlantic salmon have about 50% less total purine per surface area than their immature silver counterparts (Leclercq, unpublished data). With no changes in total purine content during European eel spawning migration, Pankhurst and Lythgoe (1982) concluded that the change from creamy-yellow to silver colour was a result of the redistribution of purines from the inner to the outer layer of the skin.

#### *Melanin and pteridines*

Variations in skin melanin and pteridine content or in the density and distribution of related chromatophores during life-stage transitions remains poorly characterized. In maturing European eel, xanthophores are lost but the density of melanophores remains unchanged (Pankhurst and Lythgoe, 1982). In nuptial Atlantic salmon, skin melanin content is also unchanged compared to immature adults. The developing grey belly would therefore appear to be a consequence of reduced total purine content and their likely inward redistribution (Leclercq, unpublished data). The formation of “egg-dummies” previously described in mature male hapochromine cichlids arises from an increased xanthophore and pteridine concentration in this area (Salzburger *et al.*, 2007). In *Xiphophorus* species, the sepiapterin group is the main pigment in the larval skin xanthophores while in adults the drosopterin group is also

synthesized increasing their overall redness (Matsumoto, 1965, Henze *et al.*, 1977).

#### **4. Physiological and endocrine control of morphological colour changes**

The genetic mechanisms controlling morphological colour change in teleosts have been recently and comprehensively reviewed with regards to melanophore genesis and apoptosis during background adaptation (proximate morphological colour change) (Sugimoto, 2002) and pigment pattern development during transition to the adult stage (ultimate morphological colour change) in zebrafish, flatfish, salmonids and vertebrates (Parichy, 2006; Hamre *et al.*, 2007, Colihueque, 2009; Mills and Patterson, 2009). The main physiological and endocrine factors of morphological colour changes reported in teleost have not been reviewed and are summarized in Table 3 and 4 along with their interactions. Their role is predominant during proximate morphological colour change but more likely secondary during ultimate morphological colour changes in front of the genetic mechanisms involved in this last phenomenon.

**Table 3.** Summary of factors and pathways leading to proximate morphological colour changes.

Type	Factor	Pathway	Chroma.	Organism	Reference
<b>• Photoprotection (UV-B photo-oxidation)</b>					
	BH4 p.-oxyd.	+Tyr	+M	Human	Schaulreuter <i>et al.</i> , 1994
	BH4 p.-oxyd.	+Tyr	+M	Frog, mouse	Wood <i>et al.</i> , 1995
		+ $\alpha$ MSH prec	+M	Rodent	Chakraborty <i>et al.</i> , 1999
		+ $\alpha$ MSHR	+M	Rodent	Bologna <i>et al.</i> , 1989
		+cGMP, +Tyr	+M	Human	Roméro-G. <i>et al.</i> , 1996
<b>• Cryptism</b>					
<i>Darkness</i>					
	+Mel	-Tyr	-M	Mouse	Valverde <i>et al.</i> , 1995
	+Mel	- $\alpha$ MSH	-M	Mouse	Weatherhead and Logan, 1981
	+Mel	-MSHc	-M	Moz. tilapia	van Eys and Wendelaar-B., 1981
	+Mel	-BH4	-M	Human	Jang, 2000
	+Mel prec	-SPR	-M	Human	Oxenkrug, 2005
<i>Dark background</i>					
	+ $\alpha$ MSHc,	+ $\alpha$ MSH, +SL	+M	Red drum	Zhu and Thomas, 1996, 1998
	+ $\alpha$ MSHc, +SLc		+M	Moz. tilapia	van Eys, 1980, 1981
	+ $\alpha$ MSH		+M	Teleosts	Abbot, 1973, Sugimoto, 2002
	$\alpha$ MSH		+M	Goldfish	Foote and Tchen, 1967
	+ $\alpha$ MSH			G. sea bream	Arends <i>et al.</i> , 2000
	+SL			Cichlid	Cánepa <i>et al.</i> , 2006
	+ACTH, +C			R. trout	Gilham and Baker, 1985
	+C			Carp	Papoutsoglou <i>et al.</i> , 2000
<i>Light background</i>					
	-MSHc		-M	Moz. tilapia	van Eys and Peter, 1981
	+MCH		-M	Flounder	Yamanome <i>et al.</i> , 2006
	+MCH		-M	R. trout	Green <i>et al.</i> , 1991
	+MCH		-M	Teleosts	Nagai <i>et al.</i> , 1986;
	+NE		-M	Teleosts	Sugimoto <i>et al.</i> , 2000, 2002
	+MCH	- $\alpha$ MSH	-M	R. trout	Baker <i>et al.</i> , 1986
	+MCH	-ACTH	-M	R. trout	Baker <i>et al.</i> , 1986
	-SL		+L, -X	Zebrafish	Fukamachi <i>et al.</i> , 2004
<b>• Communication</b>					
	+ACTH		+M	Brook trout	Premdas and Ealse, 1976b
	+ACTH		+M	Goldfish	Hama <i>et al.</i> , 1960
	+ACTH		+M	Goldfish	Egami <i>et al.</i> , 1962
<i>Competition</i>					
	+ACTH	+ $\alpha$ MSH	+M	Arctic charr	Höglund <i>et al.</i> , 2000
<i>Stressor</i>					
		+ $\alpha$ MSH		G. sea bream	Arends <i>et al.</i> , 2000
		+ $\alpha$ MSH		Moz. tilapia	Balm <i>et al.</i> , 1995
	+Slc	+SL		Salmonids	Rand-Weaver <i>et al.</i> , 1993

+: Positive effect; -: Negative effect;  $\alpha$ -MSH: Alpha-melanocyte-stimulating hormone; MSHc:  $\alpha$ MSH producing cell,  $\alpha$ MSHR:  $\alpha$ MSH receptor; ACTH: Adrenocorticotrophic hormone; BH4: Tetrahydrobiopterin; Chroma.: Chromatophore or related pigment; C: Cortisol; cGMP: Cyclic guanosine monophosphate; inhib: Inhibition; p.-oxyd.: Photo-oxidation; prec: Precursor; SL: Somatolactin; SLc: SL producing cell; SPR: Sepiapterine-reductase; Tyr: Tyrosinase; A: African; G: Gilthead; L: Leucophore; M: Melanophore; Moz: Mozambique; R.: Rainbow; X: Xanthophore.

**Table 4.** Summary of endocrine factors and pathways leading to ultimate morphological colour changes.

Type	Factor	Pathway	Effect on skin	Organism	Reference
<b>Non-nuptial Metamorphosis</b>					
	PRL	+Ss		Guppy PSp.	Tan <i>et al.</i> , 1988
	PRL	+LH		Tilapia Sp.	Rubin and Specker, 1992
	TH		+M	Flatfish	Yoo <i>et al.</i> , 2000 Hamre <i>et al.</i> , 2007
	TH		+ I/L	Brook trout	Chua and Eales, 1971
	TH		+I/L	Arctic charr	Bjerkeng <i>et al.</i> , 1999
	TSH		-E/X	Rainbow trout	Premdas and Eales, 1976b
<b>Nuptial Metamorphosis</b>					
	Ss		+M, +X	J. killifish	Niwa, 1965a and b
	Ss		+E	Arctic charr	Bjerkeng <i>et al.</i> , 1999
	BPG		-X, I/L distri	European eel	Pankhurst & Lythgoe, 1982
	Ss		+Yellowness	<i>A. burtoni</i>	Korzan <i>et al.</i> , 2008
	Ss	-HPI		Salmonids	Pottinger <i>et al.</i> , 1995, 1996 Young <i>et al.</i> , 1996 McQuillan <i>et al.</i> , 2003
	Ss	+skin $\alpha$ MSHR		Human	Scott <i>et al.</i> , 2002
	Ss	+PRL		G. sea bream	Brinca <i>et al.</i> , 2003
	Ss	+PRL		Masu salmon Psp	Onuma <i>et al.</i> , 2005
	Ss	-PRL		Masu salmon Sp	Onuma <i>et al.</i> , 2005
	Ss	+skin PRLR		G.sea bream Sp	Santos <i>et al.</i> , 2001
	Ss	+gonad PRLR		G.sea bream Adult	Cavaco <i>et al.</i> , 2003
	Ss	-gonad PRLR		G.sea bream Juvenile	Cavaco <i>et al.</i> , 2003
	GnRH	+PRL		Masu salmon in spring	Bhandari <i>et al.</i> , 2003
	PRL	+Ss		Mature male Tilapi	Rubin and Specker, 1992
	PRL	+Ss		Early Vtg Guppy	Tan <i>et al.</i> , 1988
	SL	+Ss		Coho salmon	Planas <i>et al.</i> , 1992

+: Positive effect; - negative effect;  $\alpha$ MSH: Alpha-melanocyte-stimulating hormone;  $\alpha$ MSHR:  $\alpha$ MSH receptor; BPG: Brain-pituitary-gonadal axis; **Cata**: catabolism; **distri**: distribution; **E**: Erythrophore; **G**: Gilthead; **GnRH**: Gonadotropin releasing hormone; **HPI**: hypothalamic-pituitary-interrenal; **I**: Iridophore; **L**: Leucophore; **LH**: luteinizing hormone; **M**: Melanophore; **PRL**: Prolactine; **PRLR**: PRL receptor; **PSp**: Pre-spawning; **Ss**: Sex steroids; **Sp**: Spawning; **TH**: Thyroid hormone, **TSH**: Thyroid-stimulating hormone; **Vtg**: Vitellogenic; **X**: Xanthophore.

#### 4.1. Regulation of proximate morphological colour changes

##### Primary effect of light: Mechanism of photoprotection

Light-induced melanogenesis has been most comprehensively studied in mammals, particularly in humans, but similar mechanisms can be expected in teleosts since the melanogenic pathway is highly conserved among vertebrates (Slominski *et al.*,

2004). With a significant correlation found between skin colour, total biopterins and phenylalanine hydroxylase activity, the supply of both tyrosine and BH4 would control melanogenesis. BH4 was further shown to exert a dual control on tyrosinase (Tyr).

As an essential cofactor for the activity of phenylalanine hydroxylase on phenylalanine, BH4 is the rate limiting compound in the availability of the substrate L-tyrosine which in turn regulates Tyr activity. Furthermore, BH4 directly down-regulates Tyr activity on L-tyrosine but not L-Dopa by binding to a regulatory domain of the enzyme involved in L-tyrosine conversion only. Importantly, Tyr inhibition by BH4 was effectively suppressed *in vitro* by UV-B but not UV-A through its photo-oxidation into dihydropterin and 6-biopterin which do not bind to Tyr. In humans, BH4 would therefore act as a photo-switch regulating Tyr activity and promoting *de novo* melanogenesis under UV-B photo-oxidative conditions known to induce delayed tanning (Fig.3.; Schallreuter *et al.*, 1994; Wood *et al.*, 1995; Schallreuter *et al.*, 1998; Schallreuter and Wood, 1999; Slominski *et al.*, 2004). Overall, BH4 biosynthesis, regeneration, cofactors and cellular functions were reviewed by Thöny *et al.* (2000). UV-B also seems to indirectly affect melanin synthesis. Cutaneous melanocytes of rodents exposed to UV-B increased both  $\alpha$ -MSH precursor synthesis (pro-opiomelanocortin, POMC) and  $\alpha$ -MSH receptor activity (through their redistribution to the external surface) effectively promoting melanogenesis (Bologna *et al.*, 1989; Chakraborty *et al.*, 1999). UV-B light also induced a transient increase in intracellular cGMP in human melanocytes, a potent stimulator of Tyr activity and melanogenesis (Roméro-Graillet *et al.*, 1996). Of note is that BH4 exerts a feedback inhibition on GTPCH, the first step of pteridine synthesis (Harada *et al.*, 1993).

Interestingly the binding site of BH4 on Tyr has also been identified in mushroom, frog and mouse suggesting the conservation of this mechanism across living

organisms. The adverse effect of UV-B light is arguably the most ancestral and recurrent harmful environmental factor. Despite the lack of biochemical evidence, such mechanisms of photoprotection are likely to apply in teleost species.

### **Secondary effects of light: Mechanism of background adaptation**

#### *Impact of surrounding luminosity on melanogenic factors*

During long-term background adaptation, alterations in melanophore morphology and density follow physiological colour change with the same factors involved in both phenomena (Sugimoto, 2002). The most effective pigment-cell dispersing factor, the peptide  $\alpha$ -MSH, is thought to be the driving force responsible for the increase in melanophore dendricity and density by up-regulating melanogenic gene expression (Fujii, 2000; Sugimoto, 2002). In Mozambique tilapia, exogenous  $\alpha$ -MSH was shown to increase the number and size of melanophores as well as their melanin content within 14 days (van Eys and Peters, 1981). During melanoblast differentiation, both  $\alpha$ - and  $\beta$ -MSH, but not prolactin (PRL), can stimulate *in vitro* the development of melanophores in goldfish fin tissue (Foote and Tchen, 1967; Abbott, 1973). Similarly in human epidermis,  $\alpha$ -MSH stimulates Tyr activity in melanocytes while L-tyrosine and L-Dopa regulate MSH receptor (MSHR) expression (Slominsky *et al.*, 2004). Synthesis of the neurosecretory melano-concentrating-hormone (MCH), antagonist to  $\alpha$ -MSH, is stimulated under white background and induces pigment cell aggregation but also inhibits skin melanin deposition in various teleosts (Nagai *et al.*, 1986; Green *et al.*, 1991; Sugimoto, 2002, Yamanome *et al.*, 2005, Cánepa *et al.*, 2006). However, the direct effects of MCH on melanophore or other chromatophore apoptosis are not yet demonstrated (Sugimoto, 2002).

In fish and amphibians,  $\alpha$ -MSH is secreted by the pituitary melanotropes located in the pituitary pars intermedia. In African clawed toad, melanotrope activity was shown to be controlled by suprachiasmatic-melanotrope-inhibiting neurons (SMIPs) collocated in the brain suprachiasmatic nucleus which receives direct optic input. Upon photic stimulation when animals are placed on a light background, SMIPs release three neurotransmitters (neuropeptide Y, dopamine and  $\gamma$ -aminobutyric acid) inhibiting melanotrope cell activity (Kolk *et al.*, 2002). Similarly in Mozambique tilapia it was shown, using blinded fish, that  $\alpha$ -MSH cells are not directly activated by a dark background but rather by a high ratio between direct and reflected light received by the eyes (van Eys, 1980). In tilapia and red drum (*Sciaenops ocellatus*, Sciaenidae), the proportion and activity of  $\alpha$ -MSH cells increased with increasing background darkness (van Eys, 1980; van Eys and Peters, 1981; van Eys and Wendelaar-Bonga, 1981; de Rijk *et al.*, 1990; Zhu and Thomas, 1996). Nonetheless,  $\alpha$ -MSH plasma concentrations do not increase in every teleost species exposed to a dark background (van der Salm *et al.*, 2005). This is probably due to the presence of different  $\alpha$ -MSH isoforms which may have different degrees of bioactivity depending on the species. It was in fact shown in gilthead sea bream (*Sparus aurata*, Sparidae) adapted to a white background which had significantly higher  $\alpha$ -MSH plasma concentrations than individuals exposed to a black background but their pituitary monoacetyl  $\alpha$ -MSH levels did increase after black-background adaptation (Arends *et al.*, 2000).

Collocated in the pituitary pars intermedia, somatolactin (SL) cells were stimulated together with the melanotropes during black background adaptation in Mozambic tilapia (van Eys, 1980). Red drum and *Cichlasoma dimerus* (Cichlidae) skin darkened and their plasma and pituitary SL levels increased following exposure to a



black background, low and no illumination and in blinded animals (Zhu and Thomas, 1996, 1998, Zhu *et al.*, 1999, Cánepa *et al.*, 2006). Using wild-type and SL-null mutant medaka, Fukamachi *et al.* (2004) obtained strong evidence that SL does not induce leucophore apoptosis but suppresses proliferation and morphogenesis of neural-crest derived leucophores with possibly opposite effects on xanthophores. In this experiment, background colour had no effect on the density of leucophores in SL-null mutants but a white background down-regulated SL transcription in the wild-type which exhibited an increased leucophore and a decreased xanthophore density. Exposed to a black background, the number and size of leucophores decreased but SL transcription was not up-regulated. Despite the apparent absence of an SL-melanophore relationship in medaka, a direct, time-dependant, dose-dependant and reversible effect of SL was observed on red drum melanophores *in vitro*, hence the likely presence of SL receptors. Surprisingly, high-doses of SL induced melanophore aggregation *in vitro*, in contradiction with *in vivo* observations where SL occurs at physiological levels along with other hormones (Zhu and Thomas, 1997).

Background adaptation also appears to interact with the stress axis yielding potent melanogenic function as described later. In rainbow trout, 2 weeks of dark background adaptation was shown to accelerate and increase ACTH and cortisol secretion following a stressful event (Gilham and Baker, 1985). Similarly, plasma cortisol was significantly higher in common carp adapted to a black background than to a white background (Papoutsoglou *et al.*, 2000). Furthermore, the neurosecretory melano-concentrating-hormone (MCH) was shown to inhibit the secretion of  $\alpha$ -MSH and but also ACTH in rainbow trout pituitary cells cultured (Baker *et al.*, 1986).

*Involvement of melatonin*

The pineal gland is a photosensitive organ producing melatonin which is thought to be the key player in the entrainment of circadian rhythmicity. There is also increasing evidence in the literature suggesting its involvement in background adaptation.

It has been known for a long time that melatonin has aggregating properties on melanophores in different teleost species including rainbow trout (Lerner *et al.*, 1958; Hafeez and Quay, 1970). In this respect, it was suggested that various poikilothermic vertebrates exhibit nocturnal blanching through melanophore aggregation via overnight melatonin secretion (Filadelfi and Castrucci, 1996; Fanouraki, 2007). In terms of morphological colour changes, melatonin was shown to slightly reduce Tyr activity, but more importantly to significantly inhibit  $\alpha$ -MSH-induced melanogenesis in mouse melanoma cells and hair follicles (Weatherhead and Logan, 1981; Valverde *et al.*, 1995). Similarly in Mozambique tilapia,  $\alpha$ -MSH cell activity was higher in pinealectomized individuals than in intact conspecifics only when kept in total darkness as opposed to black background. Results were confirmed by melatonin administration which induced a significant reduction in  $\alpha$ -MSH cell activity hence the conclusion that  $\alpha$ -MSH cell activity is inhibited by the action of the pineal organ in the absence of light, albeit by melatonin secretion (van Eys and Wendelaar-Bonga, 1981). Furthermore, melatonin was shown to act as an effective down regulator of BH4 biosynthesis (Jang *et al.*, 2000), a key melanogenic factor, while its precursor, N-acetyl-serotonin (NAS), is much more effective than melatonin in inhibiting sepiapterin reductase (SPR) (Oxenkrug, 2005), a key enzyme in pteridine pigment synthesis and BH4 *de novo* biosynthesis (Thöny *et al.*, 2000). Produced primarily in the pineal, the complete pathway for melatonin synthesis and degradation was recently described in mammalian skin, an extra-pineal site of melatonin synthesis (Kobayashi *et al.*, 2005; Slominsky *et*

*al.*, 2005a and b). Similarly in rainbow trout, the rate limiting enzyme for melatonin synthesis, arylalkylamine N-acetyltransferase (AANAT2), was found at the highest concentration in the skin after the pineal gland and to a lesser extent in a number of other peripheral tissues (Fernández-Durán *et al.*, 2007).

BH4 is an essential cofactor for the synthesis of peripheral eumelanin but also for the production of serotonin which is converted into melatonin in the pineal gland. In mammals, pteridine compounds (e.g. biopterin and BH4) are found at the highest concentration in the pineal gland (Fukushima and Nixon, 1980) suggesting that they may be involved in non-retinal light-dependant regulation of melatonin biosynthesis (Cremer-Bartels and Ebels, 1980). The photo-oxidizing properties of BH4, previously described with their effect on melanogenesis, could play a role in melatonin circadian biosynthesis although this has not been assessed in teleosts. In humans, BH4 is used as a marker of Seasonal Affective Disorder (SAD, “winter depression”) which is characterized by a reduced concentration of plasma BH4 and activity of the serotonergic system. Light treatment is nowadays a widely accepted therapy shown to significantly increase plasma BH4 levels and to a lesser extent tryptophan (Hoekstra *et al.*, 2003).

The pineal-chromatophore system remains poorly characterized in teleosts (Fujii, 2000). Centrally, through chronic circulating levels of melatonin, or peripherally the effect of light on the serotonergic/melatoninergic system and its cofactor BH4 might affect morphological skin pigmentation. Further research in fish is clearly needed especially given the suggested role of melatonin in the seasonal entrainment of most physiological functions (Bromage *et al.*, 2001).

**Social communication with emphasizes on melanogenicity of the stress axis**

Adrenocorticotropin hormone (ACTH) is a key factor of the HPI axis which controls the physiological stress response by stimulating cortisol release from the interrenal cells of the head kidney (Hagen *et al.*, 2006). ACTH and melanocortin peptide hormones (MSH, including  $\alpha$ -MSH,  $\beta$ -MSH and  $\gamma$ -MSH) are synthesized in the pituitary gland from the same precursor, pro-opiomelanocortin (POMC), and share a sequence responsible for their common melanogenic and pigment dispersing effect (Slominsky *et al.*, 2004). *In vitro* and *in vivo*, ACTH was shown to increase melanogenesis in the brook trout (Premdas and Eales, 1976b) and to induce melanophore differentiation from pre-existing melanoblasts in amelanotic goldfish scales (Hama *et al.*, 1960; Egami *et al.*, 1962; Loud and Mishima, 1963). In line with these findings, the key receptor regulating melanogenic activity in human and teleost skin, melanocortin 1 receptor (MC1R), has high affinity for  $\alpha$ -MSH but also ACTH (Slominsky *et al.*, 2004; Selz *et al.*, 2007). While plasma ACTH elevation appears to be a general response to all stressors tested (Sumpter, 1997),  $\alpha$ -MSH can also be excreted depending on the nature and/or the intensity of the stressor (Wendelaar-Bonga *et al.*, 1995). In sea bream, air exposure increased plasma  $\alpha$ -MSH concentration (Arends *et al.*, 2000). Therefore, beside its well known melanogenic function,  $\alpha$ -MSH could also be involved in the chronic stress response acting as a mild corticotrope (Balm *et al.*, 1995). Furthermore, non-specific environmental stress also caused rapid activation of SL-cells and increased SL plasma concentration in salmonids (Rand-Weaver *et al.*, 1993), as occurs during skin darkening from black background adaptation. Finally, L-Dopa is a common precursor of both melanins and catecholamines (dopamine, epinephrine, NE) while NE is the main neurotransmitter involved in pigment chromatophore aggregation and clearly stimulates melanophore cell death (Sugimoto *et al.*, 2000; Sugimoto, 2002).

Collectively these findings show that activation of the stress axis can induce melanogenesis directly but also stimulates melanotrope cell activity. Skin darkening is known to occur under adverse conditions in a number of teleost species, including social subordination in salmonids where it correlated with increased plasma concentration of ACTH, cortisol and  $\alpha$ -MSH in Arctic charr (O'Connor, 1999, 2000; Höglund *et al.*, 2000). Recruitment of the stress axis is likely to be the key factor of “submissive skin darkening” and to induce proximate morphological colour change in the longer term. Under other factors of morphological colour change, recruitment of the stress axis could also serve to accentuate melanin biosynthesis.

#### **4.2. Factors of ultimate morphological colour changes**

The formation of pigment pattern in teleosts at a given developmental-stage is under strong genetic control as reviewed by several authors (Parichy, 2006; Hamre *et al.*, 2007; Mills and Patterson, 2009). With a number of characterized teleost mutants, an increasing number of genes have been identified as being involved in the specification, survival, proliferation, differentiation and distribution of chromatophores toward the expression of dorsal/ventral, stripes/bar and spot patterning. The formation of adult pigment pattern originates from two distinct mechanisms: “the differentiation of metamorphic melanophores from latent precursors and/or the redeployment of embryonic/early larval melanophores”, the proportion of which vary with species (Parichy, 2006). Furthermore, local interactions (attractive and repulsive forces) between the same or different chromatophore types are also increasingly recognized as critical for pattern formation and maintenance although molecular mechanisms remain unknown (Colihueque, 2009). Gene expression is the principal factor in the development of ultimate morphological colours and environmental interactions

secondary while hormonal factors are also involved.

### **Sex-steroids: direct involvement and modulation of the stress axis**

The first clear evidence of the effect of gonadal hormones on fish nuptial colouration was obtained in Japanese killifish. Compared to mature intact controls, castration during the spawning season decreased melanophore and xanthophore density in the male but not in the female. Methyl testosterone implants led to development of male-like nuptial colouration in castrated, but not in intact, females and this was reversed by dietary administration of oestradiol ( $E_2$ ) (Niwa, 1965a and b). In Arctic charr, intra-peritoneal implants of 11-ketotestosterone (11-KT) and  $E_2$  decreased plasma testosterone (T) and increased skin carotenoid content (Bjerkeng *et al.*, 1999). Based on a mathematical model, Rajasingh *et al.* (2006) concluded that flesh depigmentation in maturing Atlantic salmon was a side effect of androgen-mediated autolytic degradation and lipid loss of the muscle. In the male *Astatotilapia burtoni*, as in other territorial harem fish (Labridae, Cichlidae), plasma levels of T and 11-KT reflect colour pattern, colour intensity and dominance status (Francis *et al.*, 1993; Semsar and Godwin, 2003; Dijkstra *et al.*, 2007; Korzan *et al.*, 2008). Evidence of the effect of sex steroids on pteridine and melanin synthesis are more limited. In European eels, hormonally induced maturation led to a reduction in xanthophore density and the redistribution of purine compounds (Pankhurst and Lythgoe, 1982). Sex steroids, mainly T and progesterone (Pg), are also involved in the development of carotenoid based nuptial colouration in lizards and birds (Cooper and McGuire, 1993; McGraw, 2006). In phrynosomatid lizards (Phrynosomatidae), T administration induced a male-like melanin density in females (Quinn and Hews, 2003). Depending on the species, sex and body area, sex steroids appear to potentially affect all pigments: carotenoids, melanin, pteridines and

purines to achieve nuptial colouration. Androgenic steroids would also control skin thickening in male fish during sexual maturation therefore inducing morphological colour change via skin structure alteration (Pottinger and Pickering, 1985).

Unsurprisingly, hormones of the BPG axis appear to be the driving force toward expression of the nuptial cover. Additionally, gonadal androgens also attenuate the stress response mediated by the HPI axis, namely ACTH and cortisol blood levels, and contribute to the sexually dimorphic stress response observed in mature salmonids (Pottinger *et al.*, 1995, 1996; Young *et al.*, 1996, McQuillan *et al.*, 2003). The reduction of basal stress from sex steroids could decrease stress-induced melanogenesis to maintain the visibility of non-melanogenic nuptial colours under low and chronic stress. Furthermore, E<sub>2</sub> increases skin pigmentation and MC1R expression, the key receptor of  $\alpha$ -MSH, in normal human melanocytes (Scott *et al.*, 2002; Thornton, 2002). In nuptial teleosts, sex steroids could also locally enhance the melanogenic effects of circulating  $\alpha$ -MSH and ACTH making experiences of acute stress quickly and highly apparent. Differences in proximate morphological response to stress between non-nuptial and nuptial cover remain to be proven in fish but would be ecologically significant as stress affects gamete quality/quantity and progeny survival (Campbell *et al.*, 1992; McCormick, 1998; Contreras-Sanchez *et al.*, 1998) while the intensity of the nuptial display is a determinant for mate selection (Bourne *et al.*, 2003; Grether *et al.*, 2005).

### **Prolactin and somatolactin alteration during life-stage transition**

Accounting for more than 300 functions in vertebrates, PRL primary function appears to be the control of cell proliferation and/or apoptosis (Manzon, 2002). In fish, PRL is primarily involved in freshwater osmoregulation by promoting the development of freshwater chloride cells and inhibiting the formation of seawater chloride cells

(McCormick, 2001). Its gene expression, synthesis and secretion are often recognized to be stimulated by low environmental osmolality (Prunet and Boeuf, 1989, Kaneko and Hirano, 1993; Taniyama *et al.*, 1999, Power, 2005; Sakamoto *et al.*, 2005). However, in sockeye salmon, gill ATPase activity and chloride cell number decreased during the spawning migration before freshwater entry and in maturing fish held in seawater, thus ahead of any osmotic cue (Shrimpton *et al.*, 2005) which might have occurred from interactions of PRL with the BPG axis. *In vitro*, PRL stimulated E<sub>2</sub> synthesis in guppy early vitellogenic oocytes (Tan *et al.*, 1988), stimulated T production by mature tilapia testis following luteinizing hormone (LH) stimulation and had no steroidogenic effects on immature tissue but inhibited their LH sensitivity (Rubin and Specker, 1992). Conversely, sex steroid feed-back on PRL synthesis and sensitivity varied according to the reproductive stage and/or season. For example in gilthead sea bream, where PRL-receptors (PRLR) are also found in the skin (Santos *et al.*, 2001), E<sub>2</sub> administration stimulated pituitary PRL secretion in winter (Brinca *et al.*, 2003) but also transcription of PRLR in gonads of maturing fish while reducing them 24-fold in juvenile gonads (Cavaco *et al.*, 2003). In primary pituitary cell culture of masu salmon, E<sub>2</sub> and 11-KT significantly increased the amounts of PRL and SL messenger RNAs (mRNAs) at the pre-spawning stage in the female (July) but halved them during the spawning period in September (Onuma *et al.*, 2005). In this species, gonadotrophin-releasing hormone analog (GnRH<sub>a</sub>) also significantly elevated PRL mRNA synthesis in spring (Bhandari *et al.*, 2003). Finally, dopamine is the primary inhibitor of both gonadotropes and PRL-releasing cells in teleosts (Ben-Jonathan and Hnasko, 2001; Dufour *et al.*, 2005). In terms of colour change, PRL has so far been involved only in physiological colour change. It weakly induced melanophore aggregation *in vitro* but effectively dispersed xanthophores and erythrophores in various teleosts (Cichlidae, Gobiidae,



Adrianichthyidae, Poeciliidae). This is particularly evident during the breeding season which, along with differential interactions of PRL with the BPG-axis according to the season and maturity status, strongly suggests its involvement in the expression of nuptial colouration (Kitta *et al.*, 1993; Oshima *et al.*, 1996; Fujii, 2000; Sköld *et al.*, 2008).

Belonging to the growth hormone/PRL family, SL physiological function has been attributed to hypercalcaemic regulation, regulation of gonadal development and regulation of energy homeostasis, particularly lipid metabolism, according to feeding regime (Kaneko and Hirano, 1993; Kakizawa *et al.*, 1993; Mingarro *et al.*, 2002, Perea, 2008) and, as previously addressed, is now clearly identified as involved in background colour adaptation (Fukamachi *et al.*, 2004). In salmonids, plasma SL increases during sexual maturation and has steroidogenic effects on gonads *in vitro* (Planas *et al.*, 1992; Rand-Weaver and Swanson, 1993; Taniyama *et al.*, 1999; Mousa and Mousa, 1999, 2000, Bhandari *et al.*, 2003) but normal sexual development occurred in SL-null medaka mutants (Fukamachi *et al.*, 2006).

Overall, PRL and SL provide interesting links between osmoregulatory ability, sexual development, alterations in feeding regime and energy metabolism which are concomitant with morphological colour change during life-stage transition in various teleosts. Long-term alteration in PRL and SL plasma concentrations during diadromous migrations and/or the breeding season are likely to be involved in ultimate morphological colour changes.

### **Thyroid hormones: a metamorphic hormone**

The role of thyroid hormone (TH), tetraiodothyronine (thyroxine, T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>) in its bioactive forms were recently reviewed with special

reference to aquaculture (Yamano, 2005). TH is best known for promoting early development and larvae-juvenile metamorphosis in teleosts; particularly in flatfish where TH has been identified as critical for the development of adult-type skin colouration, i.e. pigment cell migration and/or differentiation in various species (Yoo *et al.*, 2000; Jegstrup and Rosenkilde, 2003; Abol-Munafi *et al.*, 2005; Yamano, 2005; Shiao and Hwang, 2006; Hamre *et al.*, 2007). Using knockdown embryos for the enzyme catalyzing conversion of  $T_4$  into  $T_3$ ,  $T_3$  availability was shown to affect embryonic development of zebrafish but also pigmentation and tyrosinase mRNA expression (Walpita *et al.*, 2009). In salmonids, injection of thyroid extracts led to silvering in Atlantic salmon and brown trout (Landgrebe, 1941). Later, it was also shown that physiological increases in thyroid hormones can induce G and Hx skin deposition in the brook trout while thiourea, a thyroid inhibitor, decreased skin Hx and total purine levels (Chua and Eales, 1971). In rainbow trout, Premdas and Eales (1976b) found that thyroid-stimulating hormone (TSH) significantly increased the specific activity and levels of G and Hx, had no effect on GMP and significantly decreased IXP activity with no effects on other pteridines analysed. In Arctic charr, intraperitoneal implants releasing  $T_3$  induced skin silvering but had no effect on skin, fillet and plasma carotenoid levels (Bjerkeng *et al.*, 1999). Similarly, TH plasma level increases during parr-smolt transformation in various salmonids (Ebbesson *et al.*, 2000; McCormick *et al.*, 2003, 2007) along with gill ATPase activity, skin G concentration and skin reflectance (Haner *et al.*, 1995; Ando *et al.*, 2005). Conversely, during upstream anadromous migration TH decreases along with a reduced silver appearance, alterations in osmoregulation and sexual development (Leatherland *et al.*, 1989, Youngson and Webb, 1993).

Definitely related to life-stage metamorphosis and related pigment-cell

morphogenesis in different species, TH is to date the only factor identified as directly involved in skin purine deposition. Nonetheless, knowledge on iridophore differentiation and on the effect of TH on other pigments cells remains scarce.

## 5. Conclusion

Teleost skin colouration has strong ecological and economical significance and studies on the subject encompass a broad range of research areas such as adaptative and behavioural ecology, developmental biology, environmental disruption and even optimization of rearing systems in farmed species. By linking knowledge in those different areas, this review proposed a comprehensive framework for a broader view on the phenomenon of morphological skin colour changes in teleosts, their different types and regulating mechanisms. A key distinction was made between proximate and ultimate morphological colour changes defined respectively as the *morphological modulation of one life-stage phenotype* in response to proximate environmental cues and the *transition between two life-stage phenotypes* pre-adapted to their respective ancestral ecosystems. Primary (nutrition and UV-light) and secondary (surrounding light, photoperiod and social interactions) factors of proximate morphological colour changes were also distinguished.

Such distinctions may facilitate investigations in this field for a better understanding on the origin of a given phenotype. In practice, it highlights the need for complete environmental control when studying a specific type of morphological colour change. In farmed species, anticipation of periods when significant morphological colour changes are likely to occur is also of interest to prevent market value loss of harvested fish. For instance periods of high solar radiation occurring simultaneously with high water clarity and dark background could induce significant morphological

colour changes. Turbid freshwater run-off, unusual cloud cover and stressors can also alter skin colour beyond the range of normal variations. In ecological studies, interactions between different factors of morphological colour changes, such as stress expression according to the life-stage or interactions between habitat selection, nutrition, background colour and dominance in relation to the expression of an organism's phenotype are likely to be of behavioural and evolutionary interest.

Distinct factors and pathways of morphological colour changes often interact and occur simultaneously but also in a seasonal fashion *in vivo*. Light (intensity, spectrum, and photoperiod), water clarity and overall background reflectance but also food availability, stress factors and social interactions exhibit circannual rhythmicity in various aquatic environments. This is also true for factors of ultimate morphological colour changes. Life-stage metamorphoses, such as smoltification, and reproduction are usually seasonal events entrained by environmental cues. This is likely to induce a degree of seasonality in ultimate morphological colour changes even when complete metamorphosis does not occur. This is illustrated by a seasonal cycle of sexual development observed in immature individuals and cases of dummy-run migration of immatures toward spawning grounds (Hickling, 1935; Dodd *et al.*, 1978; de Veen, 1970; Ramsay and Witthames, 1996). Furthermore, fish have adopted a wide range of habitats, from temperate to tropical, freshwater to deep seawater, life-cycle and pigment pattern strategy. Overall, this compromises the characterization of observed morphological colour changes but the proposed framework could facilitate modeling of the different forces involved in this broad phenomenon.

As extensively described by Fujii (2000), a much diversified range of physical, chemical, humoral, neuronal and paracrine factors are known to be involved in physiological colour changes. This is often a transitional phenomenon toward

morphological colour change in the longer-term, hence the same factors are likely to be increasingly involved in both phenomena. The present review highlighted key area where knowledge is lacking in teleosts. Mechanisms of UV-protection are virtually not addressed in fish. Further investigation on the role of melatonin in skin physiology may bring valuable information on the pineal-chromatophore axis in teleost. Knowledge on background adaptation is lacking in a number of species, such as salmonids, particularly so with regards to chromatophores other than melanophores. Finally, the role of PRL, SL and TH in pigment deposition and chromatophore density remains elusive overall despite being strong candidates of ultimate morphological colour changes. In the face of the numerous factors involved and difficulties related to the biochemical or structural characterization of the skin, novel analytical tools such as molecular gene expression are likely to prove highly beneficial in this field for example to quantify pigment biosynthetic pathways and hormone receptors in the integument under different environmental conditions and during life-stage transitions.

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**CHAPTER 3**  
**GENERAL MATERIALS AND METHODS**

## CHAPTER 3 GENERAL MATERIALS AND METHODS

### 1. Experimental animals and facilities

#### 1.1. Commercial trials (*Chapter 4 and 5*)

Experiments performed within the commercial facilities of the industrial partner (Marine Harvest (Scotland) Ltd.) used commercial strains of Atlantic salmon post-smolt stocked at sea between February and April (S0+; S1). Four sea-sites were located in the same Loch system (Loch Linnhe, Lat: 56°39', Long: -5°19') and one site in Loch Sunart (56°41', -5°42'), all of them within a 10 miles radius. Fish were reared under full commercial management in square cages exposed to natural thermo-cycle. The photoperiod manipulation regime was standard among pens and conforms to routine industry practice. Continuous artificial-light was applied from early January to June during the second-year at sea using wide-spectrum lighting-systems (metal-halide) positioned in a standard manner among pens and sites. This photoperiod manipulation strategy was used as control in Chapter 4 where alternative lighting-windows and technologies were tested as detailed in the corresponding sections. The fish were fed a commercial diet according to manufacturer recommendations adjusted daily to estimated biomass and observed feeding response using automated computer controlled feeding systems. Sampling was performed on-farm or at the primary processing plant (Blar Mhor, Fort William, Scotland) where single pen harvest batches from the above mentioned sites were delivered in the form of whole fish bled.

## 1.2. Triploidy studies (*Chapter 6*)

The parental origin, fertilization and triploidization protocols are thoroughly described in the corresponding chapter (Chapter 5). Triploidy was induced 300°C min post-fertilisation (30 min at 10°C) by applying an hydrostatic pressure shock (65,500 kPa, 5 min; Johnstone and Stet, 1995) using a customized pressure chamber connected to an air compressor. Triploidization of newly fertilised eggs by hydrostatic pressure shock is effective, well established and the most commonly used method to induce sterility in Atlantic salmon (Benfey and Sutterlin, 1984; McGeachy *et al.*, 1995; Benfey, 2001; Cotter *et al.*, 2002; Oppedal *et al.*, 2003; Piferrer *et al.*, 2009). This pressure shock prevents the second meiotic division, i.e. extrusion of the second polar body at the single cell stage, resulting in a triploid zygote yielding one paternal and two maternal sets of haploid chromosomes. Physical as opposed to chemical induction is overall the most successful and, pressure as opposed to temperature shock is more reliable when treating large eggs in large volumes (Piferrer *et al.*, 2009).

## 2. Sampling procedures

### 2.1. Anaesthesia and euthanasia

Animals were anaesthetised following a 24h starvation period prior to any experimental procedure. Commercial stocks were anaesthetised in a bath of tricaine methanesulphonate (30 ppm; MS-222; Alpharma, Fordingbridge, England; Fig.1). Experimental fish were anaesthetised in a bath of 2-phenoxyethanol (100 ppm; Sigma-Aldrich, Poole, UK). In all instances, anaesthetic baths were made fresh at frequent intervals. Loss of equilibrium was always induced within a 3 min period and full recovery was observed within 5 min in clean aerated water when fish were returned to



the original housing. Post-sampling mortalities were exceptional and typically below 0.1%.

When sampled at the primary processing plant, fish were previously slaughtered under standard commercial practices. At harvest, the stock was pumped from a single sea-cage to a well-boat and transported live under controlled conditions to the harvest station (Mallaig, Scotland). Slaughtering was performed using compressed air spiking causing instantaneous physical destruction of the brain and immediately followed by exsanguination by cutting two to four gill-arches. Harvested biomass was kept in ice-slurry during bleed-out and transferred to the primary processing plant where sampling was performed ahead of evisceration. Aside from the commercial channel or within non-commercial facilities, animals were sacrificed in accordance with the Home Office regulations by an anaesthetic overdose followed by strong blow to the cranium causing instantaneous and lethal brain concussion.

## **2.2. Blood sampling (*Chapter 4 and 5*)**

Blood was withdrawn from the caudal vein of anaesthetised or sacrificed fish using 1 or 2 mL sterile syringes fitted with 25G, 23G or 21G sterile hypodermic needles (Terumo N.V.; Leuven, Belgium) according to fish size (Fig.1d). Fitted syringes were previously flushed with a 4 mg.mL<sup>-1</sup> solution of ammonium heparin salt (Sigma-Aldrich, Poole, UK) to prevent blood coagulation. The blood was stored on ice in 1.5 mL Eppendorf tubes, centrifuged (1200 g; 15 min; 4°C) and the plasma aliquoted into 0.5 mL Eppendorf tubes then stored at -70°C until analysis.



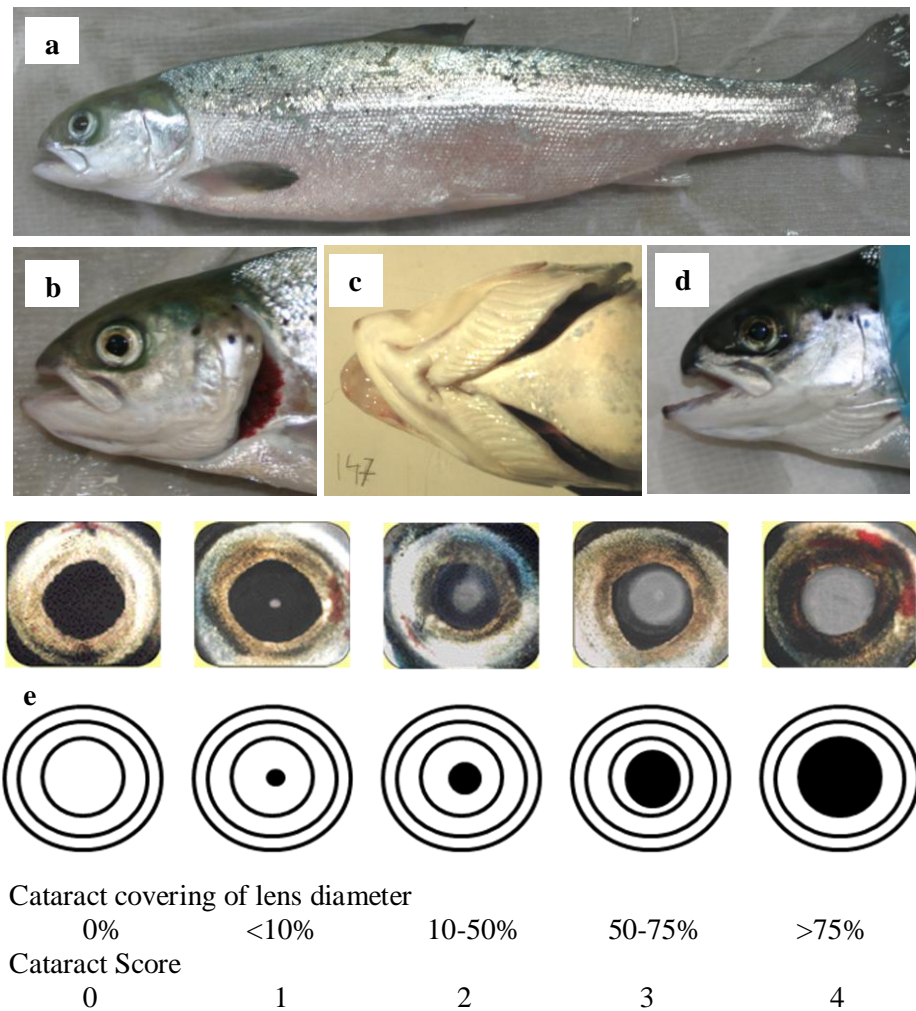
**Figure 1** Illustration of sampling procedures as performed within the commercial facilities. (a.): Biomass crowding for random population sampling. (b.) Fish under anaesthesia showing loss of equilibrium (c.) sampling area and (d.) blood withdrawal from the caudal vein of anaesthetised Atlantic salmon.

### 2.3. Body-size, deformity assessment and tissue sampling

#### Body-size and deformity

Whole body wet weight (BW) was determined using an electronic balance (Model QC7DCE-S,  $\pm 5$  g or Model BFS-242-020C,  $\pm 0.1$  g; Sartorius AG, UK) and fork-length (FL;  $\pm 1$  mm) using a customised measuring board. Sacrificed fish were sexed, gonad weight (GW) and in one trial heart weight (HW; *Paper VIII*) were measured using an electronic balance (Model Scout Pro SPU202,  $\pm 0.001$ g; Ohaus C<sup>TP</sup>, Nanikon, Switzerland). Average BW, as determined by the farm staff following standard commercial practices (batch sample weight with a total of  $n \sim 120$  fish/pen

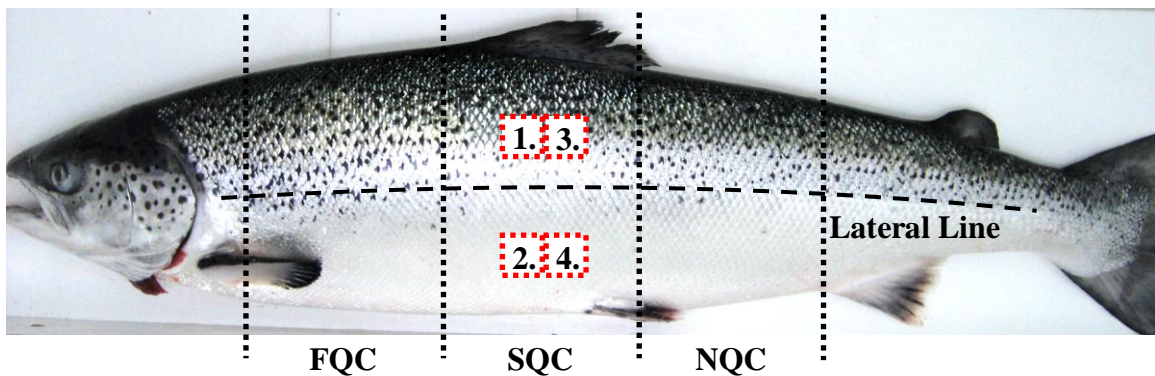
measured in 12 to 15 batches), was occasionally used and acknowledged (*Paper V*). Assessment of observable external deformities, ocular cataract and jaw morphometry are described in the corresponding experimental chapter (*Chapter 6*). Example of observed external deformities are provided (Fig.2) along with the macroscopic cataract scoring scale used (Wall and Richard, 1992; Bass and Wall, 2004; Fig.2e). Whole carcasses were frozen at -20 °C for later radiological analyses of vertebral deformities.



**Figure 2.** Example of gross external deformities: (a.) Spinal deformity in the tail area, (b.) Left-hand side opercular shortening, (c.) Twisted lower jaw (d.) Upper jaw shortening and (e.) Atlantic salmon cataract scoring scale (from Bass and Wall, 2004).

### Flesh quality and skin sampling (*Chapter 4*)

The left-hand side fillet was cut with the standardized Scottish quality cut (SQC; Fig.3) removed, placed into a sealed polypropylene bag and stored at -20°C until quality analysis. Dorsal and ventral skin samples were taken from standardized areas of the left-hand side SQC (Fig.3) using a scalpel in order to remove all the flesh but no “white materials” (*Stratum argenteum*, Hawkes, 1976) from the integument. They were divided in two vertically (for carotenoids-melanin and purine pigment analyses), individually wrapped in foil and sealed in polypropylene bags stored at -20°C until analysis. For the skin colour reconditioning trial, both hand-side fillets of 5 silver and 5 intermediary coloured fish, as subjectively determined, were cut into the standardized Flesh, Scottish and Norwegian quality cut (6 cuts/fish; Fig.3), rinsed with distilled water to prevent blood contamination and used for skin colour analysis (*Paper IV*).



**Figure 3.** Localization of the standardized flesh quality and skin samples used for analysis.

**FQC:** Flesh quality cut, **SQC:** Scottish quality cut, **NQC:** Norwegian quality cut. **1.** and **2.:** Dorsal and ventral skin sample respectively excised for carotenoid and melanin pigment analysis. **3.** and **4.:** Dorsal and ventral skin sample respectively excised for purine pigment analysis.

## Tissue preservation

Transverse sections from the middle area of ovarian tissues were preserved in an excess of Bouin's fixative (Bios Europe, Skelmersdale, UK) or 10% buffered formalin (Table 1) for histological analysis of ovarian development (*Chapter 4 and 5*). Whole hearts and whole left hand-side gill apparatus were preserved in 10% buffered formalin for later assessment of tissue morphometry; enucleated eyes were preserved in Bouin's fixative for later histological analysis (*Chapter 6*).

**Table 1.** Formulation of 10% buffered formalin used for tissue fixation.

<b>Chemical</b>	<b>Unit</b>	<b>Quantity</b>
Deionized water	(mL)	900
Disodium hydrogen phosphate	(g)	6.5
Sodium dihydrogen phosphate	(g)	3.5
Formalin (40% formaldehyde)	(mL)	100

Gill biopsy (*Chapter 6*) were performed immediately after fish sacrifice by removing the tip (~2-3 mm) of 4 to 6 primary gill filaments from the left-hand side second gill arch using surgical fine tipped scissors; a procedure which has no detrimental effect on subsequent survival, growth and salinity tolerance (McCormick, 1993). The tissue was placed into a 1.5 mL eppendorf containing 100  $\mu$ L of ice-cold SEI buffer (See Table 11 for composition), immediately frozen on liquid nitrogen and stored at -70°C until analysis of Gill Na<sup>+</sup> K<sup>+</sup> ATPase activity used as an indicator of the parr-smolt transformation.



### 3. Body-size, growth and deformity analysis

#### 3.1. Fulton condition factor (K) and weight-length relationship (WLR)

Fulton condition factor (K; equation 1) is primarily a shape indicator derived from the cube law (Froese, 2006) that positively correlates in Atlantic salmon with “visual fatness score”, i.e. rounded appearance, dietary energy fed (Young *et al.*, 2006), total flesh lipid at the parr stage (Herbinger and Friars, 1991) and filleting yield (Einen *et al.*, 1998, 1999). It is accordingly a recognized indicator of nutritional status.

**Equation 1:**

$$K = 100 \frac{BW}{FL^3}$$

Where: BW is the whole body wet-weight (g) and FL the fork length (cm).

Weight-length relationships (WLR; *Paper III*) are historically used in fisheries to estimate an individual BW from its FL. Interestingly it describes the relative variations in individuals' shape (K) over the population size range being overall constant when the WLR is isometric ( $b = 3$ ). In contrast, heavier individuals have a higher K in populations exhibiting a positive allometric WLR ( $b > 3$ ) and inversely a smaller K in populations with a negative allometric WLR ( $b < 3$ ). The accepted form of WLR in fish (Equation 2) was transformed into its logarithmic equivalent (Equation 3) to determine the parameters of the relationship by least-square regression using Microsoft Excel software (Keys, 1928; Froese, 2006).

**Equation 2:**  $BW = a FL^b$

**Equation 3:**  $\log (BW) = \log (a) + b * \log (FL)$

Where: BW (g) and FL (cm) are body-size variables and a and b are parameters of the relationship.

To avoid bias from the uneven fish distribution over the population size range, WLR was calculated by pooling individuals per 1 cm FL classes and determining the mean-BW for each length class (Froese, 2006). Using the b-parameter from WLR analyses at each sampling point, mean-b  $\pm$  SE was also determined for individual sea-sites. This parameter was suggested to better represent the actual WLR of a species (Froese, 2006). It is arguably a better indicator of the relative shape of fish achieved on average in individual harvest batches from a given site.

### 3.2. Body-size dimorphism (*Paper III*)

Dimorphisms in BW, FL and K between genders at an immature stage and between cohorts of sexual development were calculated within single pen harvest-batch (sibling fish of identical age) according to equation 4 (Saillant *et al.*; 2001).

**Equation 4:**

$$Adv(X_D) = 100 \frac{(X_D - X_d)}{X_d}$$

Where: Adv ( $X_D$ ) is the size advantage (%) in parameter X (BW; FL or K) of the dominant cohort D;  $X_D$  is the mean-value of parameter X in the dominant cohort (D) and  $X_d$  is the mean-value of parameter X in the dominated cohort (d).

### 2.3.3. Round weight gain, specific growth rate and food conversion ratio (*Chapter 5, 6*)

The relative weight gain (RWG) was calculated according to equation 5

**Equation 5:**

$$RWG = 100 \frac{(BW_2 - BW_1)}{BW_1}$$

Where:  $BW_1$  and  $BW_2$  are mean-whole body wet weight (g) at times  $t_1$  and  $t_2$  respectively

The average daily BW gain was expressed as specific growth rate (SGR) using equations 6 and 7 (Taylor *et al.*, 2006).

**Equation 6:**

$$SGR = 100 (e^{g-1})$$

Where: SGR is expressed in  $\% \cdot \text{day}^{-1}$  and  $g$  is the instantaneous growth rate calculated according to equation 7

**Equation 7:**

$$g = \frac{(\ln BW_2 - \ln BW_1)}{(t_2 - t_1)}$$

Where:  $BW_1$  and  $BW_2$  are mean-whole body wet weight (g) at times  $t_1$  and  $t_2$  respectively and  $(t_2 - t_1)$  is expressed in days.

Biological food conversion ratio (FCR) is a measure of how efficiently the feed distributed to the stock is converted into body mass therefore constituting a good performance indicator of the feeding strategy applied. This ratio does not take into account uneaten food such that, despite the standardization of feeding rate to standing biomass and water temperature, it must be interpreted with caution when assessing the biological efficiency of feed conversion into body mass. FCR was calculated using farm data according to equation 8.

**Equation 8:**

$$FCR = \frac{\text{Weight of dry food used}}{\text{Weight gain of biomass}}$$



Where: Weight of biomass gain over the period is based on the estimated number of fish and sample-weight performed at the start and at the end of the period.

### **3.4. Organ morphometry (Chapter 6)**

#### **Jaw**

Pictures of the anterior body-portion were used to assess jaw morphometry by image analysis (Image ProPlus 4.5, Mediacybernetics, MD) according to Lijalad and Powell (2009). To do so, the length of the upper and lower jaws were determined by measuring the distances between the base of the pectoral fin and the tip of the upper and lower jaws respectively (Fig.4a). The lower jaw index was then calculated as follow (Equation 9):

#### **Equation 9**

$$LJI = \frac{L1}{L2}$$

Where: LJI is the lower jaw index, L1 and L2 are the length (expressed in the same unit) of the upper and of the lower jaw respectively.

#### **Gill apparatus**

The left-hand side gill apparatus stored in 10% buffered formalin was rinsed in distilled water. The second gill arch (from the outer surface) was carefully dissected, laid flat on a copy stand and photographed (Eos 350D digital camera, Canon Europe L<sup>td</sup>, Uxbridge, UK) with a calibrating scale. The length of the gill arch bone, the total surface of the second gill arch apparatus, the total number of primary filaments and the length of every 5 primary filaments was measured by image analysis (Fig.4b).

**Heart**

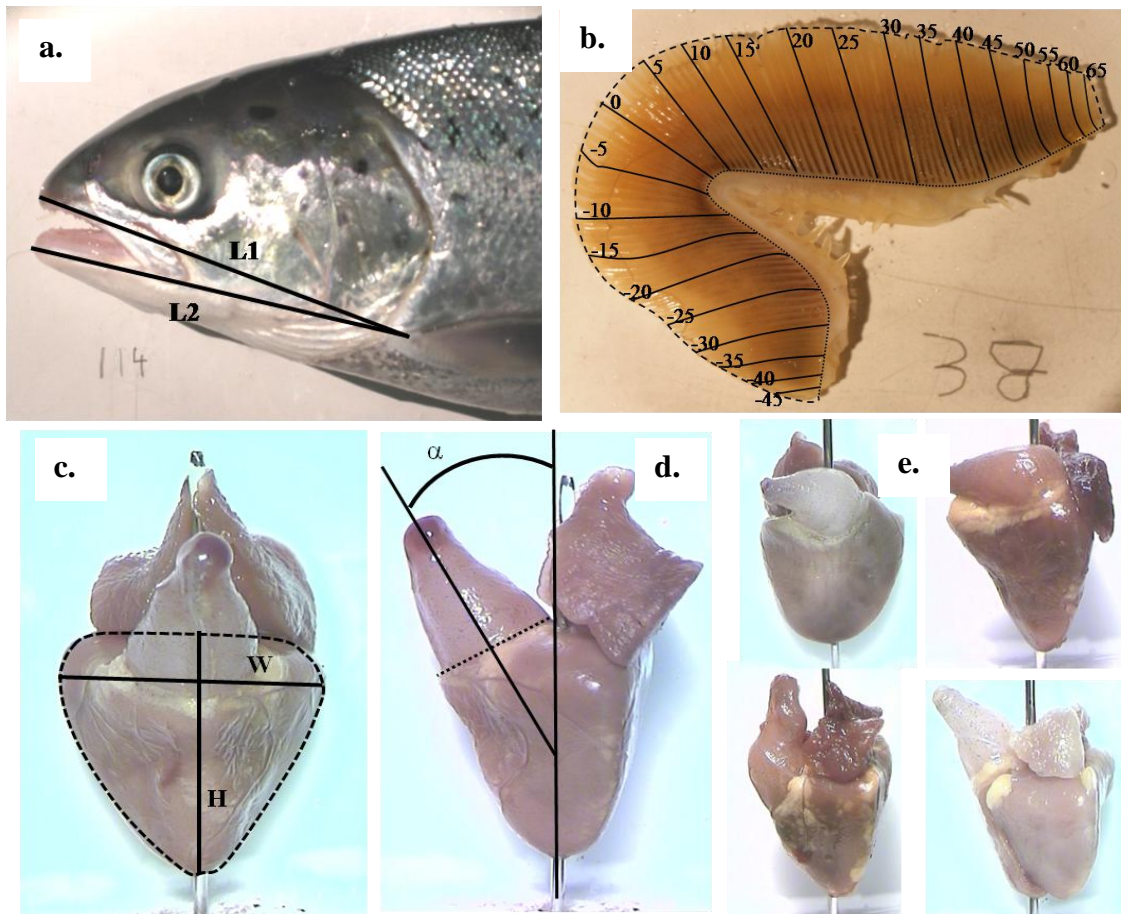
The cardio-somatic index (CSI), representing the weight of the heart relative to the whole body weight of the organism, was calculated according to equation 10.

**Equation 10:**

$$CSI = 100 \frac{HW}{BW}$$

Where: HW and BW are the heart and body wet-weight (g) respectively and CSI is expressed in %.

Heart morphometry was determined according to Poppe *et al.* (2003) by using the height:width ratio and the alignment of the bulbus arteriosus as shape indicators. The height:width ratio was determined by image analysis of the heart pictured with the cranio-ventral surface uppermost. The ventricle height (H) was defined as the distance from the apex to the middle of the ventricle base and the ventricle width (W) as the widest segment of the ventricle parallel to its base (Fig.4c). The alignment of the bulbus arteriosus (angle  $\alpha$ ) was determined from the same hearts pictured in lateral recumbency. The longitudinal axis of the heart was defined as passing through the apex and the middle of the ventricle base where the bulbus arteriosus and the ventricle joint. A second line was defined as passing through the middle of the bulbus arteriosus and the angle ( $\alpha$ ) at the intersection of both lines was measured.



**Figure 4.** Assessment of organ morphometry by image analysis (a.) Length of the upper and lower jaws (L1 and L2 respectively) used to determine the lower jaw index (LJI) (b.) Left-hand side second gill-arch showing the length of the gill arch bone (short-dot), the surface of the arch apparatus (area within the dotted lines), the length (black line) and number of selected primary filaments. Heart pictured (c.) with the cranio-ventral surface uppermost to measure the ventricle height (H) and width (W), (d.) in lateral recumbency to measure angle  $\alpha$  (e.) Example of gross heart deformities.

#### 4. Maturation assessment (*Chapter 4 and 5*)

Maturation status was determined based on gonado-somatic index (GSI), gonad histology, plasma testosterone level and/or the occurrence of external nuptial colouration depending on sex and calendar-time.

#### 4.1. Gonado-somatic-index

The GSI, calculated according to equation 11, represents the weight of the gonads relative to the BW of the organism. Males were classified as immature or sexually recruited based on the bimodal GSI frequency distribution in the population with a threshold value of 0.2% (Kadri et al., 1997a; Taranger *et al.*, 1998).

**Equation 11:**

$$GSI = 100 \frac{GW}{BW}$$

Where: GW and BW are the whole gonad and body wet weight (g) respectively and GSI is expressed in %.

#### 4.2. Ovarian histology

##### Tissue preparation, processing and sectioning

Transverse ovarian sections were preserved in Bouin's fixative for 24h-36h then rinsed and stored in 70% ethanol or kept in 10% buffered formalin until processing for histological analysis. Two discrete sections per ovary sample were cassetted and processed overnight in an automatic tissue processor (Model Shandon citadelle 2000; Thermo Fisher Scientific, Astmoor Runcorn, UK) according to Table 2.

**Table 2.** Processing of fixed gonadal tissue for histology.

Steps	Chemical bath	Duration (min)
1	50% Methylated spirit	30
2	80% Methylated spirit	90
3; 4; 5	100% Methylated spirit	90; 90; 90
6; 7	100% Absolute ethanol	105; 90
8; 9	Choloroform	50; 50
10; 11; 12	Molten wax	105; 90; until cassetting

Ovarian tissues were then embedded in wax, refrigerated on a cold plate until wax hardening, exposed using a motorized electronic microtome (Shandon finesse;

Thermo Fisher Scientific, Astmoor Runcorn, UK) set-up for sectioning at 20  $\mu\text{m}$ , hydrated in distilled water (~20 min, room temperature) and refrigerated on a cold plate (< 5 min, -10°C; RA Lamb; Astmoor Runcorn, UK). The tissue block was then sectioned at a 5  $\mu\text{m}$  thickness, floated onto distilled water maintained at 40°C, mounted on microscopic slides (Surgipath, Bretton, UK) and stored in a Windsor incubator (55°C; Agar Scientific; Stansted, UK) until staining.

### Histological staining

Sections were stained with Mayer's haematoxylin and eosin Y following a protocol modified from Bancroft and Stevens (1990; Table 3).

**Table 3.** Mayer's haematoxylin and eosin Y histological staining protocol.

Steps	Chemical bath	Duration (min)
1; 2	Xylene	3; 2
3	Absolute ethanol	2
4	Methylated spirit	1
5	Running tap water	0.5 to 1
6	Haematoxylin	5
7	Running tap water	1
8	1% Acid alcohol	4 quick dips
9	Running tap water	0.5 to 1
10	Scotts tap water substitute	1
11	Running tap water	0.5 to 1
12	1:8 (1% Eosin (aq) : Putt's Eosin)	5
13	Running tap water	0.5 to 1
14	Methylated spirit	1
15; 16	Absolute alcohol	2; 1
17; 18	Xylene	5; Until cover slipping (Pertex mountant)

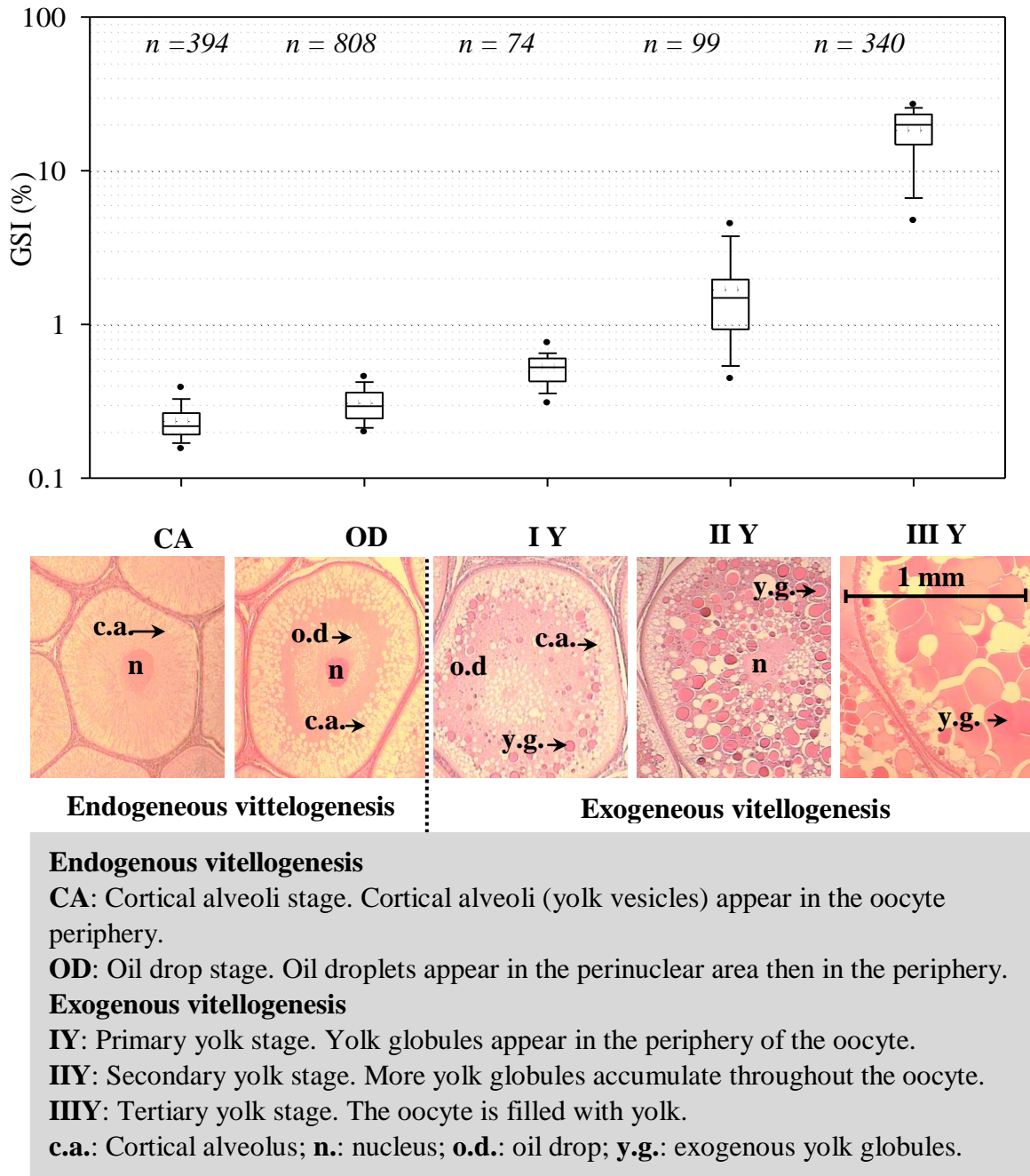
### Analysis

Mounted sections were examined under light microscopy (Model Olympus BH-2; Olympus Optical Co., London, UK) connected to a zoom lens (18-108/2.5; Olympus Optical Co., London, UK). Ovaries were classified based on the developmental stage of

their leading oocyte cohort (Taranger *et al.*; 1999; Fig.5). Females were deemed as immature up to the oil drop stage (endogenous vitellogenesis), as initiating maturation at the primary/secondary yolk stages (exogenous vitellogenesis indicating the onset and commitment toward maturation) and as sexually advanced when the tertiary yolk stage was observed. Females with a GSI over ~10% and an oocyte diameter over ~ 3mm could not be analysed histologically due to the excess of yolk preventing section integrity. They were classified at the tertiary yolk stage from external observation.

#### **4.3. Plasma-testosterone analysis**

Plasma-testosterone (T) level was determined using an indirect competitive radioimmunoassay (RIA) method modified from Duston and Bromage (1987). In this technique, a known amount of tritiated-testosterone ( $^3\text{H-T}$ ) radiolabel is added to the sample containing an unknown amount of naturally present T. An excess of T-antibody is then added for competitive binding with labelled and naturally present T-antigens. Uncomplexed T-antigens are then neutralized and washed away such that the quantity of  $^3\text{H-T}$  radiolabel-antibody measured is inversely proportional to the quantity of T naturally present in the sample.



**Figure 5.** Key to the classification of oocyte developmental stage in 2 sea-winter female Atlantic salmon (Taranger *et al.*, 1999) given with the range of gonadosomatic-index (GSI) measured for ovaries at the corresponding oocyte leading stage (measured from June to December from *n* fish given in italics). The dashed line represent the sample mean. The box-plot represents the distribution of the values with, starting from the upper mark, the 95<sup>th</sup> (dot), 90<sup>th</sup>, 75<sup>th</sup> (upper quartile), 50<sup>th</sup> (median), 25<sup>th</sup> (lower quartile), 10<sup>th</sup> and 5<sup>th</sup> (dot) percentile represented.

### Plasma-T extraction

Plasma-T was extracted by suspension of the plasma organic phase in a solvent. To do so, a 200  $\mu\text{L}$  plasma aliquot was transferred to a polypropylene assay tube (LP3P; Thermo Life Sciences, Basingstoke, UK) to which 1 mL ethyl acetate (BDH Chemicals Ltd; Poole, UK) was added before spinning on a rotary mixer for 1h at room temperature. The mixture was then centrifuged (430 g; 10 min; 4°C) and the supernatant transferred into a clean polypropylene tube stored capped at 4°C until analysis.

### Assay and charcoal buffers

The assay buffer was made prior to the day of the assay (stored at 4°C overnight) and the charcoal buffer freshly prepared prior use using AnalaR grade chemicals (Table 4).

**Table 4.** Composition of the (a) assay and (b) charcoal buffers used in the T ssay.

Chemical	Unit	Quantity
<b>(a) Assay buffer composition (stir at 50°C for 30 min minimum)</b>		
Nanopure water	(mL)	250
Disodium hydrogen phosphate	(g)	4.44
Sodium dihydrogen phosphate	(g)	2.91
Sodium chloride	(g)	2.25
Gelatin	(g)	0.25
<b>(b) Charcoal buffer composition (stir on ice for 30 min minimum)</b>		
Assay buffer	(mL)	100
Charcoal	(g)	5.0
Dextran	(g)	4.9

### <sup>3</sup>H-T-radiolabel and T-antibody

A primary stock solution (“Hot stock”) of tritiated-T, ([1,2,6,7-<sup>3</sup>H]-T; Amersham Biosciences; Little Chalton, UK) was supplied in quantities of 37 MBq or 1 mCi (4 mL at at 9.25 MBq.mL<sup>-1</sup> or 250  $\mu\text{Ci.mL}^{-1}$ ). An intermediate radiolabel stock solution (“Stock A”) was prepared by diluting 20  $\mu\text{l}$  of the primary stock in 2 mL of



AnalaR grade absolute ethanol and stored at  $-20^{\circ}\text{C}$  in a high performance glass vial (Packard Bioscience, Little Chalfort, UK). From this, a working radiolabel solution was freshly prepared for each assay by diluting  $\sim 50\ \mu\text{L}$  of the intermediate stock in 10 mL assay buffer to give an activity of  $\sim 10,000\ \text{dpm}/100\ \mu\text{L}$ .

A working T-antibody solution was made immediately prior use by adding 10 mL of distilled water per vial of freeze dried rabbit antitestosterone antiserum (CER groupe, Marloie, Belgium) according to manufacturer recommendations.

### **T-standard stock solution and curve, line standards and controls**

An intermediate T-standard stock solution was prepared by dissolving 1 mg of dry T (Sigma-Aldrich, Poole, UK) in 10 mL of absolute ethanol ( $100\ \mu\text{g}\cdot\text{mL}^{-1}$ ) which was then diluted by a factor 100 (0.1 mL in 9.9 mL absolute ethanol;  $1\ \mu\text{g}\cdot\text{mL}^{-1}$ ) then by a factor 10 (1 mL in 9.9 mL absolute ethanol;  $100\ \text{ng}\cdot\text{mL}^{-1}$ ) before storage at  $-20^{\circ}\text{C}$  in a 20 mL high performance glass vial. Immediately prior each assay, a working T-standard solution of  $10\ \text{ng}\cdot\text{mL}^{-1}$  was prepared by diluting 0.1 mL of the intermediate standard solution in 0.9 mL absolute ethanol. This was used to prepare the T-standard curve by serial dilutions (in duplicates; Table 5). A T-line standard was also prepared by diluting 0.2 mL of the intermediate T-standard stock solution in 9.8 mL of absolute ethanol as to obtain a T-line standard concentration of  $2\ \text{ng}\cdot\text{mL}^{-1}$  ( $100\ \text{pg}\cdot 50\mu\text{L}^{-1}$ ; Table 5). This was stored at  $-20^{\circ}\text{C}$  in a 20 mL high performance glass vial and used over all assays to assess reproducibility of measurements within and between assays (4 line standards analysed per assay).

**Table 5.** Standards and control used in the testosterone radioimmunoassay.

Tube n°	Standard ( $\mu\text{L.tube}^{-1}$ )	100% ethanol ( $\mu\text{L.tube}^{-1}$ )	Standard amount ( $\text{pg.tube}^{-1}$ )
<b>(a) Testosterone standard curve</b>			
1; 2	100 of working standard solution <sup>1</sup>	0	1000
3; 4	100 of working standard solution <sup>1</sup>	100	500
5; 6	100 of 3; 4	100	250
7; 8	100 of 5; 6	100	125
9; 10	100 of 7; 8	100	62.5
11; 12	100 of 9; 10	100	31.3
13; 14	100 of 11; 12	100	15.6
15; 16	100 of 13; 14	100	7.8
17; 18	100 of 15; 16	100	3.9
19; 20 <sup>2</sup>	100 of 17; 18	100	1.95*
21; 22	0	100	0
<b>(b) Control (Non specific bindings ; NSB)</b>			
23; 24	0	100	0
<b>(c) Line standard</b>			
25; 26	50 $\mu\text{L}$ of Line standard	0	100
X; Y <sup>3</sup>	50 $\mu\text{L}$ of Line standard	0	100

<sup>1</sup> Working T-standard solution concentration:  $10 \text{ ng.mL}^{-1}$ ; <sup>2</sup> Remove 100  $\mu\text{L}$  from tubes 19 and 20; <sup>3</sup> X and Y are the last tubes of the assay.

### Assay protocol

All standards, controls and samples were assayed in duplicate, except line standards in quadruplicate (Table 6).

**Table 6.** Testosterone radioimmunoassay protocol.

Step	Day 1
1	Create assay buffer
2	Create T-standard stock solution and T-standard curve
3	Create T-line standard
4	Add 200 $\mu\text{L}$ of plasma extract to LP3P tubes in duplicate ( <i>start at tube n°27</i> )
5	Dry down all LP3P tubes in vacuum rotary evaporator ( $< 35^\circ\text{C}$ ; 45 min; Genevac Ltd.; Suffolk, UK) and cool to $4^\circ\text{C}$
6	Create T-antibody solution
7	Create $^3\text{H-T}$ radiolabel
8	Add 100 $\mu\text{L}$ T-antibody solution to all LP3P tubes except NSB's to which add 100 $\mu\text{L}$ of assay buffer
9	Add 100 $\mu\text{L}$ of $^3\text{H-T}$ -radiolabel to all LP3P tubes
10	Vortex and incubate ( $4^\circ\text{C}$ ; 18 h)

**(Table 6 Cont.) Day 2**

- 11** Create charcoal buffer
- 12** Add 500 µL of charcoal buffer to all LP3P tubes
- 13** Vortex, incubate (4°C; 15 min) and centrifuge (770 g; 15 min; 4°C)
- 14** Transfer 400 µL of supernatant to scintillation vials (6 mL polyethylene; Packard Bioscience; Groningen, Netherlands).
- 15** Add 100 µL of <sup>3</sup>H-T radiolabel to two clean scintillation vials  
(Totals used to calculate total radioactivity)
- 16** Add 4 mL of scintillation fluid (Ultima Gold; Packard Bioscience; Groningen, Netherlands) in all scintillation vials and in a clean one (Blank used to calculate background radioactivity)
- 17** Vortex all scintillation vials and count radioactivity in a scintillation counter (1900TR LSA, Canberra Packard Ltd.; Pangbourne, UK)  
(Count blank for 10 min; all other for 2 min)

**Note:** Plasma-T of mature fish was analysed using 50µL of plasma extract due to high T-concentration.

**Calculations**

The standards and samples disintegrations per minute (dpm) values were converted by Riasmart software (Canberra Packard; Pangbourne, UK) into percentage <sup>3</sup>H-radiolabel bindings using blank, totals and NSBs dpm values. This was then converted by the software into total T content (pg.tube<sup>-1</sup>) based on the standard curve of percentage <sup>3</sup>H-T-radiolabel binding for known amount of total T content (pg.tube<sup>-1</sup>; Fig.6). The total T content was subsequently converted into plasma T concentration (pg.mL<sup>-1</sup>) in the corresponding sample using Microsoft Excel software according to equations 12 and 13.

**Equation 12**

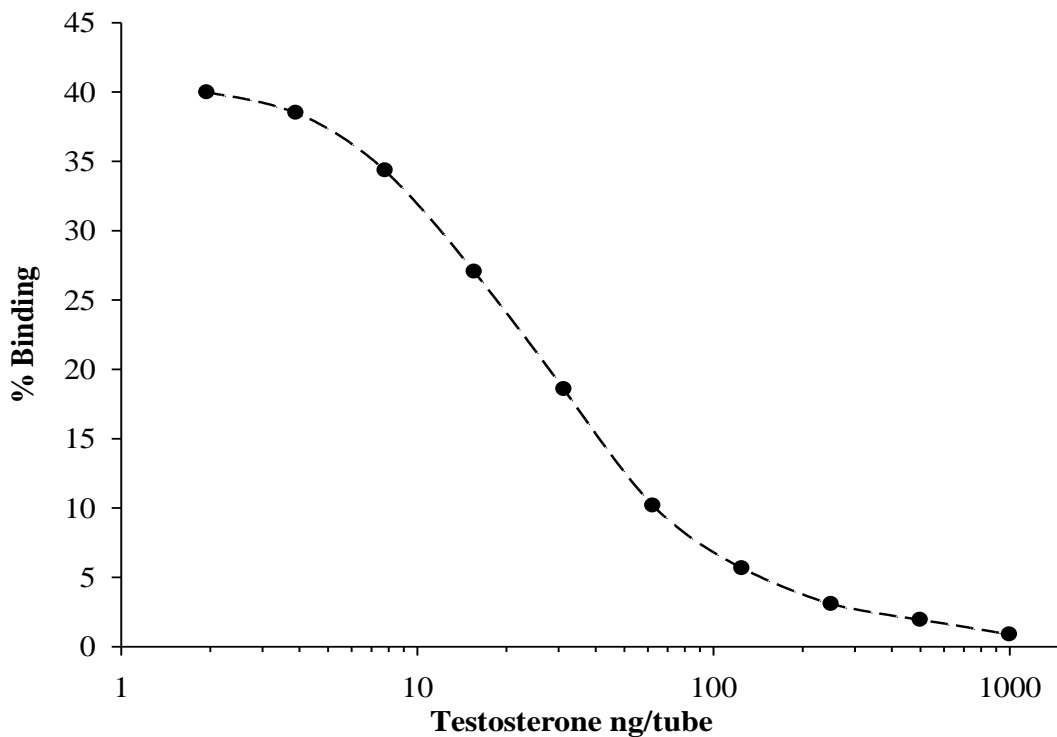
$$Total\ T_{(Extract\ assayed)} = Total\ T_{(Assay\ tube)} * \frac{Volume\ (Total\ supernatant)}{Volume\ (Supernatant\ assayed)}$$

Where: Total T in the assay tube is expressed in pg per total volume of extract assayed (200 µL or 50 µL) and, in our protocol, Volume<sub>(Supernatant assayed)</sub> = 400 mL (step 14); Volume<sub>(Total supernatant)</sub> = 700 mL (step 8, 9 and 12).

**Equation 13**

$$[T_{Plasma}] = \left[ \left( \frac{Total\ T_{(Extract\ assayed)}}{Volume_{(Extract\ assayed)}} \right) * Volume_{(Total\ plasma\ extract)} \right] * DF$$

Where: Plasma-T concentration  $[T_{plasma}]$  is expressed in  $pg.mL^{-1}$  and, in our protocol,  $Volume_{(Extract\ assayed)} = 200\ \mu L$  or  $50\ \mu L$ ;  $Volume_{(Total\ plasma\ extract)} = 1200\ \mu L$  ( $1000\ \mu L$  ethyl acetate +  $200\ \mu L$  of plasma); DF (Plasma dilution factor) = 5 ( $1000\ \mu L$  ethyl acetate /  $200\ \mu L$  of plasma).



**Figure 6.** Typical standard curve from a testosterone radioimmunoassay.

The total T content in the extract assayed was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

**Quality control and validation**

The sensitivity of the assay, *i.e.* the minimum amount of T statistically distinguishable from zero, was  $1.9\ pg.tube^{-1}$ . Quality control performed with the T-line

standard (100 pg.tube<sup>-1</sup>) in quadruplicate was used to check the reproducibility of measurements. The intra-assay coefficient of variation was  $8.95 \pm 1.55\%$  and the inter-assay coefficient of variation was 8.52%.

#### **4.4. Skin colour prevalence**

The display of nuptial cover was confirmed as a reliable indicator of advanced sexual development in both males (GSI>1%) and females (III<sup>ty</sup> yolk stage) and from August to December. It was then used over this calendar-period to estimate maturation rate in a large proportion of harvested stock. Their prevalence was determined by a minimum of 600 observations from at least three counting sessions over the batch processing period.

### **5. Flesh quality analysis (*Paper IV*)**

Flesh quality was analysed at the accredited 'Fish Health and Quality Laboratory' of Marine Harvest (Scotland) Ltd (Lochailort, UK) according to commercial protocols used for post-harvest quality control. Prior to analysis, SQC sample was thawed overnight under lightproof cover at 4°C, skinned and deboned.

#### **5.1. Roche colour card score**

The flesh colouration was scored subjectively against a standardized colour card scale (Roche *SalmoFan*<sup>TM</sup> lineal colour card for salmonids, scale 20-34; Hoffman-LaRoche Ltd.; Basel, Switzerland) by the same two independent assessors throughout the study period (Robb, 2001). To do so, flesh samples were placed into a neutral grey colour cabinet fitted with a D65 noon-daylight bulb (6500 °K) which closely reproduce the colours as would be observed under natural light conditions. Flesh colour was

scored in the dorsal, midline and ventral regions by placing the colour card in the vicinity of the corresponding area and selecting the closest colour score.

## **5.2. Colorimetric analysis**

The flesh colour was further described instrumentally in the same three body areas using a tristimulus colorimeter (Minolta Chroma Meter, CR-310, Minolta Corporation; Osaka, Japan) calibrated against a standard calibration tile (number 12133332) (Robb, 2001). This apparatus directs a pulse of light at the surface of the sample and measures the composition of the reflected light according to the CIE (1976)  $L^*a^*b^*$  colour space (Robb, 2001). This colour space describes all the colours visible by the human eye using three coordinates or primary parameters: The colour lightness ( $L^*$ -value), red-green chromacity ( $a^*$ -value) and yellow-blue chromacity ( $b^*$ -value).

## **5.3. Total pigment, lipids and PUFAs level**

The whole SQC was then homogenized using a food processor and a sub-sample was placed into the cup cell of a near infrared reflectance (NIR) analyser (FOSS-6500 NIR analyser, Foss NIRSystems, Foss UK Ltd.; Didcot, UK) for analysis of total carotenoid content ( $\text{mg.kg}^{-1}$ ), total lipid content (% sample) and total polyunsaturated fatty acids (PUFAs; % lipids). This apparatus measures the reflectance of near-infrared light (700 to 2500 nm) directed to the sample to determine its biochemical composition using predictive equations (Fjellanger *et al.*, 2001). The sample cell was washed and dried before each use. The NIR analyser was calibrated daily prior to analysis using a calibration check cell (number 20635). The predictive equations were controlled twice over the experimental period by comparing the NIR output against primary analytical

methods (Soxhlet and HPLC analyses for total lipid and total pigment level respectively; Skretting Aquaculture Research Centre; Stavanger, Norway).

## **6. Pigment analysis (*Paper IV*)**

### **6.1. Sample preparation**

On the day of the analysis, wet skin samples were weighed ((Model AE100,  $\pm$  0.0001 g; Mettler Toledo, UK) and their surface area determined by image analysis (Image ProPlus 4.5, Mediacybernetics, MD) using a colour digital video camera coupled to a light microscope (Model BH-2; Olympus Optical, London, UK) equipped with an MTV-3 adapter (Olympus Optical Company, Ltd., Tokyo, Japan) and connected to a PC. Skinned SQCs and gonads were thawed overnight, homogenized and a ~1 g subsample was separated and weighed before extraction.

### **6.2. Carotenoid pigment analysis**

#### **Pigment extraction**

The protocol for carotenoid pigment extraction was common among tissues (Table 7; modified from Barua *et al.*, 1993 as described in Torstensen *et al.*, 2005).

**Table 7.** Protocol for extraction of carotenoid pigments from flesh, gonad and skin

Step	
1	Add 10 mL ethyl acetate:ethanol (1:1, v/v) in a 50 mL stopper glass tube containing the tissue sample
2	Homogenize the tissue with an Ultraturrax tissue disrupter, rinse the probe in clean ethyl acetate:ethanol and dry between tubes
3	Centrifuge (220 g; 5 min)
4	Transfer the supernatant in a clean 35 mL stopper glass tube, cap and shed
5	Resuspend tissue pellet in 5 mL ethyl acetate (vortex)
6	Centrifuge (220 g; 5 min)
7	Transfer and pool supernatants
8	Repeat steps 5 to 7 in 5 mL iso-hexane (Use skin pellet for melanin extraction)
9	Evaporate pooled supernatants to dryness on a nitrogen evaporator
10	Place dried supernatants in a vacuum dessicator overnight in darkness
11	Redissolve extracts in 5 to 20 mL iso-hexane
12	Transfer a 2 mL fraction into a 2 mL Eppendorf
13	Centrifuge (6100 g; 5 min) before injection into the HPLC system

### Standards preparation and HPLC system

Three carotenoid standards were used (asthaxanthin, cantaxanthine and  $\beta$ -carotene; DSM Nutritional Products, Basle, Switzerland) and prepared as follows: Approximately 0.75 g of the compound (crystalline form) was weighed and transferred to a 50 mL volumetric flask by rinsing the boat with 2.5 mL chloroform. The flask was then made up to volume with iso-hexane and mixed. A 1.25 mL fraction of this solution was transferred into a clean 25 mL volumetric flask supplemented with 1 mL chloroform and made up to volume with iso-hexane. The concentration of this working standard solution was determined spectrophotometrically at 450 nm (CE 2021; Cecil Instruments, Cambridge, UK) according to equation 14. The HPLC system used for carotenoid pigment analysis is detailed in the corresponding chapter (Chapter 3.2).



### Calculation

The concentration of carotenoid compounds was calculated using Microsoft Excel software according to equation 14 for standards, equation 15 for skin samples and equation 16 for flesh and gonad samples.

#### Equation 14

$$[\text{Standard } X] = 10,000 \frac{\text{Abs}(X)_{450 \text{ nm}}}{E(X)_{1\%}^{1\text{cm}}}$$

Where: the standard concentration in compound X ([Standard X]) is expressed in  $\mu\text{g.mL}^{-1}$ ,  $E(X)_{1\%}^{1\text{cm}}$  is the percent extinction coefficient of the carotenoid standard X with a value of 2,100 for Ax; of 2,200 for Cx and of 2,300 for  $\beta$ -carotene.

#### Equation 15

$$[\text{Skin } X] = \frac{([\text{Standard } X] * X_{\text{peak area}} * DV)}{(\text{Standard}_{\text{peak area}} * \text{Sample surface area})}$$

Where: the concentration of pigment compound X in the skin sample ([Skin X]) is expressed in  $\mu\text{g.cm}^{-2}$ ; the standard concentration in compound X ([Standard X]; equation 12) is expressed in  $\mu\text{g.ml}^{-1}$ ; the sample surface area in  $\text{cm}^2$  and the sample dilution volume (DV; Table 7 step 11) in ml.

#### Equation 16

$$[\text{Sample } X] = \frac{([\text{Standard } X] * X_{\text{peak area}} * DV)}{(\text{Standard}_{\text{peak area}} * \text{Sample weight})}$$

Where: the concentration of carotenoid compound X in the sample ([Sample X]) is expressed in  $\mu\text{g.g}^{-1}$ ; the standard concentration in compound X ([Standard X]; equation 14) is expressed in  $\mu\text{g.mL}^{-1}$ ; the sample weight in g and the sample dilution volume (DV; Table 7 step 11) in mL.

### Quality control

The intra-assay coefficient of variations in standard peak areas were, all tissues considered, 2.08% for  $\beta$ -carotene, 1.69% for Cx and 1.72% for Ax. The inter-assay coefficient of variation in standard peak areas were not calculated as the method used external standards prepared frequently and of concentration measured spectrophotometrically for each assay. The coefficient of variations in elution time of the standards were 2.87% ( $\beta$ -carotene at 1.778 min), 3.33% (Cx at 5,276 min) and 5.08% (Ax at 12.511 min) and, for the samples, 1.32% ( $\beta$ -carotene at 1.811 min), 1.02% (Cx at 5.408 min) and 1.46% (Ax at 12.643 min).

### 6.3. Melanin pigment analysis

Skin melanin was extracted from the carotenoid stripped skin pellet (Table 7, step 8; Table 8).

**Table 8.** Protocol for extraction of melanin from carotenoid stripped skin pellet.

Step	
1	Resuspend the skin pellet in 10 mL 0.2% NaOH and store overnight at 4°C
2	Vortex and boil the suspension in 0.2% NaOH for 1h with frequent mixing
3	Centrifuge (2100 g; 5 min)
4	Transfer supernatant in a PP-test tube 5 (Cellstar®, Frickenhausen, Germany)
5	Repeat steps 2 and 3 and pool supernatants
6	Add 0.2% NaOH into pooled supernatants to 20 mL precisely and vortex
7	Transfer a 2 mL fraction of the total supernatant into a 2 mL Eppendorf
8	Centrifuge (6100 g; 5 min) before reading on a spectrophotometer at 340 nm

### Standards preparation

A melanin standard stock solution of 1 mg.mL<sup>-1</sup> was prepared by dissolving 5 mg of dry sepia synthetic melanin (Sigma-Aldrich, Poole, UK) in 5 mL of 1 M NaOH : 3% H<sub>2</sub>O<sub>2</sub> mixture (100:1; v/v) in a boiling water bath for 30 min (Szisch *et al.*, 2002). A

working melanin standard solution of  $0.1 \text{ mg.mL}^{-1}$  was prepared in triplicate by dilution of the stock solution to a factor 10 in the same NaOH : H<sub>2</sub>O<sub>2</sub> mixture. From each melanin standard stock solution, a melanin standard curve was prepared by serial dilution to a factor 2 (Fig.7). The average coefficient of variation between triplicate standards was 7.12%.

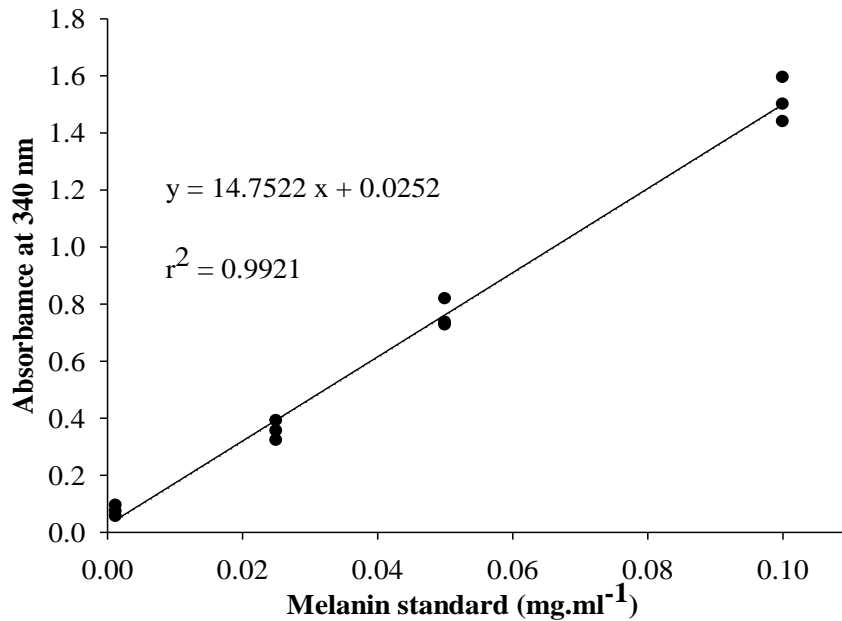
### **Spectrophotometry and calculation**

Melanin extracts were measured spectrophotometrically at 340 nm (CE 2021; Cecil Instruments, Cambridge, UK; Szisch *et al.*, 2002). The melanin concentration within an extract was obtained from the intersect of the sample absorbance at 340 nm with the standard curve red at the same wavelength (Fig.7). The melanin content in the sample extract ( $\text{mg.cm}^{-2}$ ) was calculated according to equation 17.

#### **Equation 17**

$$[\text{Skin melanin}] = \frac{([\text{Extract melanin}] * DV)}{\text{Sample surface area}}$$

Where: the concentration of melanin in the skin sample ([Skin melanin]) is expressed in  $\text{mg.cm}^{-2}$ ; the concentration of melanin in the extract ([Extract melanin]) in  $\text{mg.mL}^{-1}$ ; the sample surface area in  $\text{cm}^2$  and the sample dilution volume (DV = 20 mL in our protocol) in mL.



**Figure 7.** Standard curve of the sepia synthetic melanin standard shown with the linear regression line and equation used for determination of melanin concentration in skin samples. The total melanin content in the sample extract was determined by intersecting the standard curve at the point corresponding to the sample absorbance.

#### 6.4. Purine pigment analysis

##### Pigment extraction

The protocol for extraction of purine pigments was modified from Oliphant (1987a; Table 9).

**Table 9.** Protocol for extraction of purine pigment as modified from Oliphant (1987a).

Step	Day 1
1	Add 10 mL 0.1 M NaOH in a 50 mL stopper glass tube with the skin sample
2	Homogenize with an Ultraturrax tissue disrupter, rinse the probe in clean 0.1 M NaOH and dry between tubes
3	Place the glass tube in an ultrasonic bath (20 min), vortex regularly (40 min)
4	Centrifuge (220 g, 5 min)
5	Transfer the supernatant in a clean foiled 50 mL stopper glass tube and cap
6	Repeat twice steps 3 to 5 in 5 mL 0.1 M NaOH and pool supernatants
7	Resuspend skin pellet in 10 mL 0.1 M NaOH (vortex) and cap
8	Store the tissue solution and pooled supernatants overnight at 4°C
	Day 2
9	Confirm translucence of the last supernatant and centrifuge (220 g, 5 min)
10	Pool the last supernatant (Total supernatants volume = 30 mL) and vortex
11	Transfer a 2 mL fraction of the total supernatants to a 5 mL glass vial
12	Add 2 mL distilled water in the 5 mL glass vial and vortex
13	Transfer a 2 mL fraction into a 2 mL Eppendorf
14	Centrifuge (6100 g; 5 min) before injection into the HPLC sstem

### **Standards preparation and HPLC system**

Purine standards (guanine (G), hypoxanthine (Hx) and adenine; Sigma-Aldrich, Poole, UK) were dissolved in 0.05 M NaOH at concentrations of 0.5 mg.mL<sup>-1</sup> (adenine) and 1 mg.mL<sup>-1</sup> (G, Hx). Working standard solutions were prepared by serial dilution to a concentration of 1 µg.mL<sup>-1</sup> prior to injection into the HPLC system. Pteridine standards (xanthopterin, sepiapterine, leucopterin, xanthine, biopterin, isoxanthopterin, lumazine; Schircks Laboratories, Jona, Switzerland) were dissolved according to manufacturer recommendations in 0.05 M NaOH at concentrations ranging from 0.2 to 1 mg mL<sup>-1</sup>. Serial dilutions to concentrations of 1, 2 or 5 µg.mL<sup>-1</sup> were performed prior to injection into the HPLC system. The HPLC system used for purine pigment analysis is detailed in the corresponding chapter (Chapter 3, paper IV). Preliminary analyses revealed that only G and Hx were present in our samples such that, for each daily set of sample analysed, only G and Hx standards were injected in triplicate.

### **Calculations**

The concentration of G and purine compounds in the skin (µg.cm<sup>-2</sup>) was calculated according to equation 15 using external standards at known concentration.

### **Quality control**

The inter-assay coefficient of variation in standard peak area was 10.19% for Hx and 9.45% for G. The intra-assay coefficient of variation in standard peak area was 5.42% for Hx and 4.66% for G. The coefficient of variations in elution times were 8.25% (Hx standard at 7.06 min), 8.02% (G standard at 8.11 min), 7.12% (Hx sample at 7.12 min) and 5.93% (G sample at 8.12 min).

## **7. Skin colour reconditioning (*Paper IV*)**

### **7.1. Treatment preparation**

Ice of four different salinities was prepared by dissolving NaCl salt to concentrations of 0 M; 0.25 M; 0.5 M and 0.75 M NaCl in tapwater to mimic industrial conditions. Freshwater ice supplemented with caffeine (Sigma-Aldrich, Poole, UK) was also prepared at a concentration of 5 mM which is known to induce melanophore dispersion (Rodionov *et al.*, 1998; n = 5 ice treatments). Prior to use, the ice blocks were crushed and placed in individual household plastic containers (~ 40 x 40 x 15 cm). The different ice baths were labelled and placed on ice in a styrofoam fish box to maintain ice temperature throughout the experiment.

### **7.2. Colorimetric analysis**

Immediately after fish dissection and before treatment application (T0), the skin colour composition of each standardized cuts (n = 6 cuts/fish with 5 silver and 5 intermediary coloured fish used) was measured instrumentally in triplicates in the dorsal and in the ventral area then averaged per body area. To do so, a tristimulus colorimeter (Minolta Chroma Meter, CR-310, Minolta Corporation; Osaka, Japan) calibrated against a standard calibration tile (number 12133332) (Robb, 2001) was used as previously addressed for analysis of flesh colour. Each cut was then randomly assigned to an ice treatment and the last cut was placed in an empty container in direct contact with skin by juxtaposing another sample. Skin colour was then assessed using the same methodology at T0 + 6 h, T0 + 24 h and T0 + 30 h. Results showed that a\*-value (red-greenness) and b\*-value (yellow-blueness) were close to achromatic in the dorsal area. In such conditions, minor changes in those parameters leads to high and irrelevant

changes in hue ( $H_{ab}$ ) and chroma ( $C_{ab}$ ; Doolan *et al.*, 2008) which were therefore not analysed.

## 8. Smoltification assessment (*Chapter 6*)

The parr-smolt transformation was assessed by regular assessment of the fish silvering index and Gill  $Na^+$ ,  $K^+$ -ATPase activity and ultimately by sea-water challenge.

### 8.1. Silvering index

Fish silvering index was scored according to a previously published scoring scale (Sigholt *et al.*, 1995; Table 10)

**Table 10.** Silvering index scoring scale for smoltification assessment.

Silvering Index	Description
1	Parr
2	Some silvering, parr mark visible
3	Fully silver but parr mark visible
4	Smolt, no parr mark visible

## 8.2. Sea-water challenge

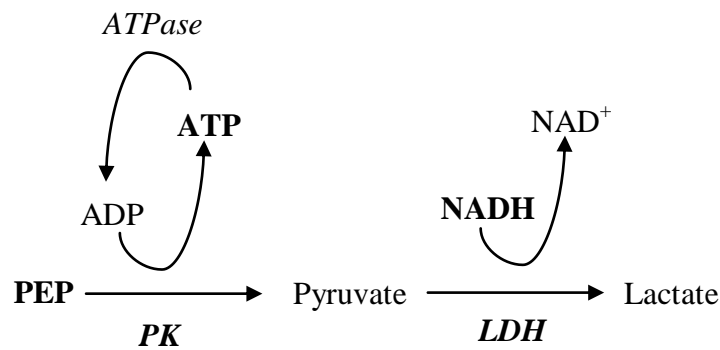
Smoltification was ultimately confirmed by challenging the stock in seawater (n = 5 fish/replicate/family/ploidy) a few days prior to potential seawater transfer. The challenge was conducted for 96 h in 35 ppt artificial seawater aerated and maintained at 10°C. The occurrence of mortalities during the challenge was recorded.

## 8.3. Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity analysis

Gill Na<sup>+</sup>, K<sup>+</sup>, ATPase is an ion-translocating enzyme found at high concentration in teleost chloride cells where it is used for osmoregulation in seawater (salt secretion). The activity of this enzyme is therefore commonly used as an indicator of parr-smolt transformation in salmonids (McCormick, 1993).

Gill Na<sup>+</sup>, K<sup>+</sup>, ATPase activity was determined using a coupled kinetic assay according to McCormick (1993). The ATPase assay (Fig.8) is based on the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) by pyruvate kinase (PK) which yields one molecule of pyruvate and of adenosine triphosphate (ATP). This reaction is coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH) which is concomitant with the oxidation of one molecule of the coenzyme NADH (reducing agent) into NAD<sup>+</sup> (nicotinamide adenine dinucleotide). The enzymatic hydrolysis of ATP into ADP by ATPase is therefore coupled to the enzymatic oxidation of NADH with the oxidation of 1 molecule of NADH corresponding to the production of 1 molecule of ADP by the enzyme ATPase.





**Figure 8.** Schematic representation of the coupled chemical reactions occurring when performing the ATPase activity assay. Chemicals shown in *italic* are enzymes, in **bold** are supplied in the assay medium.

This assay measures the kinetic rate of NADH oxidation into NAD<sup>+</sup>, which is directly proportional to the ATPase activity in the sample, by determining the reduction in the sample absorbance at 340 nm in the absence and presence of ouabain, an ATPase inhibitor. Indeed, NADH but not NAD<sup>+</sup> has a peak absorbance at this wavelength. Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity is then expressed in  $\mu\text{mol ADP} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$  by measuring the total protein content in the volume of gill homogenate assayed for ATPase activity using a Bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, Poole, UK) (McCormick, 1993; Greene and Henikoff, 1996).

### **Buffer, solutions and standards preparation**

The following assay constituents were prepared in advance of samples analysis and stored as described in Table 11.

**Table 11.** Buffers, solutions and standard used in the Gill Na<sup>+</sup>, K<sup>+</sup>, ATPase activity assay.

<b>Chemical</b>	<b>Unit</b>	<b>Quantity</b>
<b>(a) SEI buffer composition (store at 4°C for 3 months)</b>		
Nanopure water	(mL)	475
Sucrose	(g)	25.67
Sodium EDTA	(g)	1.86
Imidazole	(g)	1.70
Nanopure water	(mL)	Make up to 500
1.0 M hydrochloric acid		Adjust to pH 7.3 with
<b>(b) SEID buffer (store at room temperature for 1 week)</b>		
SEI buffer	(mL)	20
Sodium deoxycholic acid	(g)	0.1
<b>(c) Imidazole buffer (store at 4°C for 3 months)</b>		
Nanopure water	(mL)	475
Imidazole	(g)	1.702
Nanopure water	(mL)	Make up to 500
1.0 M hydrochloric acid		Adjust to pH 7.5
<b>(d) Salt buffer (store at 4°C for 3 months)</b>		
Imidazole buffer	(mL)	475
Sodium chloride	(g)	5.52
Hydrous magnesium chloride	(g)	1.07
Potassium chloride	(g)	1.57
Nanopure water	(mL)	Make up to 500
<b>(e) PEP (store in 5 mL aliquots at -70°C)</b>		
Imidazole buffer	(mL)	100
Phosphoenolpyruvate	(g)	0.491
<b>(f) Ouabain solution (store in darkness at room temperature for 3 months)</b>		
Imidazole buffer	(mL)	50
Ouabain	(g)	0.382
<b>(g) Sodium acetate buffer</b>		
Nanopure water	(mL)	100
Sodium acetate	(g)	0.4627
1.0 M hydrochloric acid		Adjust to pH 6.8
<b>(h) ADP Standard stock (store in 200 µL aliquots at -70°C)</b>		
Sodium acetate buffer	(mL)	25
Adenosine diphosphate	(g)	0.0489

### ATPase activity

The assay medium was prepared on the day of use in a volumetric column and supplemented with imidazole buffer or ouabain solution then with salt buffer prior to use (Table 12).

**Table 12.** Composition of the assay mediums used in the Gill Na<sup>+</sup>, K<sup>+</sup>, ATPase assay.

<b>Chemical</b>	<b>Unit</b>	<b>Quantity</b>
<b>(a) Assay medium (Immediately prior to use)</b>		
Pyruvate kinase	( $\mu$ L)	8.2
Lactate dehydrogenase	( $\mu$ L)	11.5
NADH	(mg)	5
PEP	(mL)	5
ATP	(g)	0.0145
Imidazole buffer	(mL)	Make up to 35
<b>(b) AM-I (Immediately prior to use in 20 mL glass vials)</b>		
AM	(mL)	17.5
Imidazole buffer	(mL)	1.25
<b>(c) AM-O (Immediately prior to use in 20 mL glass vials)</b>		
AM	(mL)	17.5
Ouabain solution	(mL)	1.25
<b>(d) AM-I Salt (In 20 mL glass vials; store at 4°C until use)</b>		
AM-I	(mL)	8.1
Salt buffer	(mL)	2.7
<b>(e) AM-O Salt (In 20 mL glass vials; store at 4°C until use)</b>		
AM-O	(mL)	8.1
Salt buffer	(mL)	2.7

Ouabain solution (Ouabain octahydrate, Sigma-Aldrich, Poole, UK). Pyruvate kinase (from rabbit muscle, Sigma-Aldrich, Poole, UK) and lactate dehydrogenase (Type II solution from rabbit muscle, Sigma-Aldrich, Poole, UK) is centrifuged (6000 g; 8 min; 5°C) and the supernatant only is used.

The ADP standard curve (Fig.9a) was prepared immediately prior use by diluting the ADP standard stock (Table 11h) in 1.5 mL Eppendorfs as shown in Table 13.

**Table 13.** ADP standard curve preparation for use in the Gill Na<sup>+</sup>, K<sup>+</sup>, ATPase assay.

<b>Imidazole buffer (<math>\mu</math>L)</b>	<b>ADP Standard stock (<math>\mu</math>L)</b>	<b>ADP Standards (nmol.10<math>\mu</math>L<sup>-1</sup>)</b>
200	0	0
175	25	5
150	50	10
100	100	20

Samples were then processed as follow (Table 14) to quantify Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity.

**Table 14.** Protocol for gill homogenate preparation and determination of their Na<sup>+</sup>, K<sup>+</sup>-ATPase activity.

Step	
1	Transfer 10 µL of each ADP standards in triplicate to wells on a 96 flat-well multiwell plate
2	Thaw 21 gill biopsy samples thoroughly
3	Add 25 µL SEID to each sample
4	Homogenise each sample using a motorized pestle
5	Centrifuge (2200 g; 0.5 min; 4°C)
6	Warm AM-I Salt and AM-O Salt in a water bath at 26°C for 10 min
7	Transfer 10 µL of each sample in quadruplicate to the assay plate
9	Add 200 µL of warmed AM-I Salt medium to wells of all ADP standards and of two replicates per sample
10	Add 200 µL of warmed AM-O Salt medium to wells of the remaining 2 replicates per sample
11	Measure the oxidation of NADH over 10 min at 340 nm using a multiwell spectrophotometer (Multiskan Ex., Labsystems, Finland) equipped with an halogen optic lamp (8V, 50W, > 500°C; OSRAM, München, Germany)
12	Refreeze sample at -70°C for later determination of protein content

Note: The procedure must be completed within 30 min as enzyme activity decline after homogenization of the gill tissue.

### Protein determination

The protein standard curve (Fig.9b) was prepared immediately prior use by diluting 2 mg.mL<sup>-1</sup> of bovine serum albumin (BSA) standard (Protein standard 2 mg BSA/mL; Sigma-Aldrich, Poole, UK) in 1.5 mL Eppendorfs (Table 15).

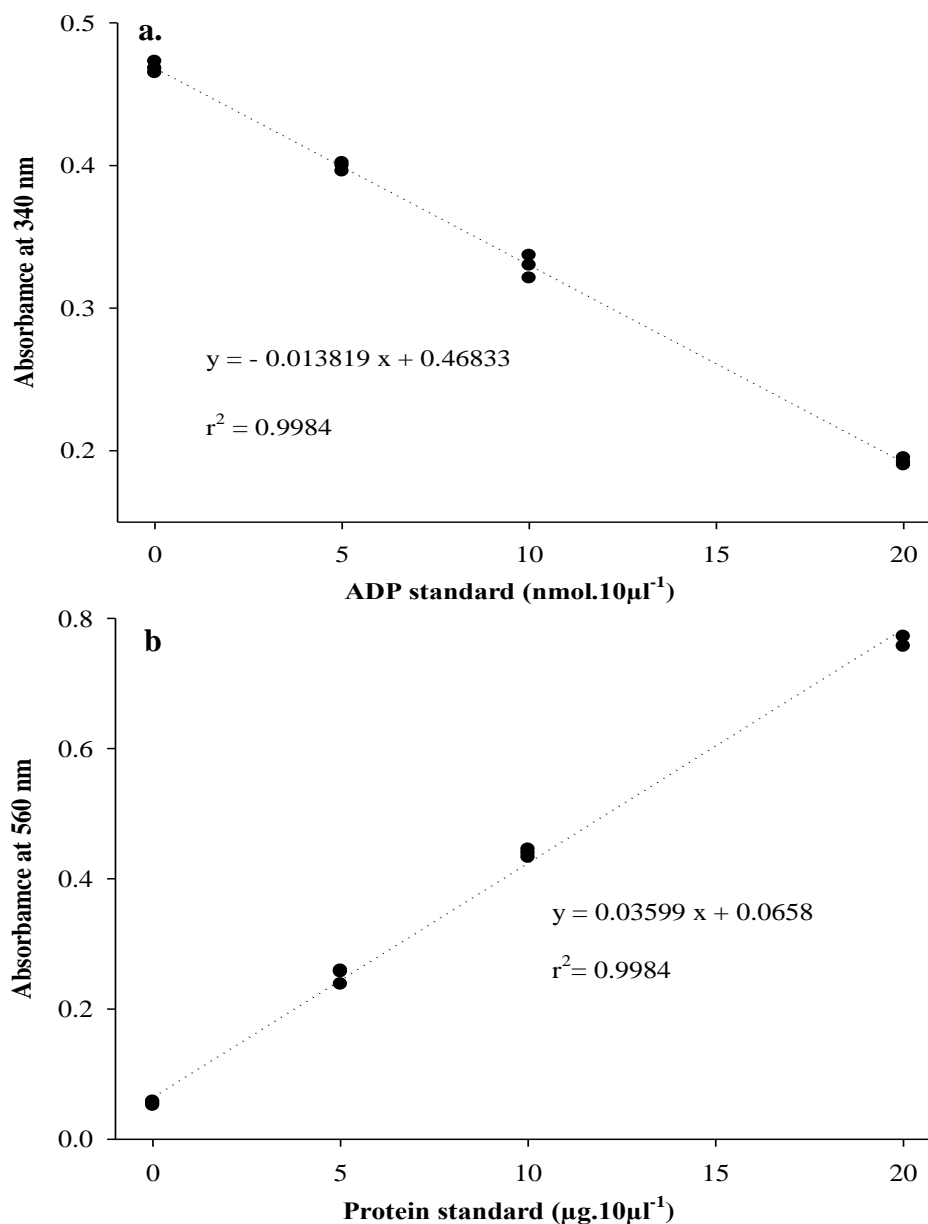
**Table 15.** Protein standard curve preparation for use in the Gill Na<sup>+</sup>, K<sup>+</sup>, ATPase assay.

2 mg.mL <sup>-1</sup> BSA	Distilled water (µL)	Standard protein content (µg.10µL <sup>-1</sup> )
0	100	0
25	75	5
50	50	10
100	0	20

The total protein content in the volume of gill homogenate assayed for Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (10µL) was determined according to the following protocol (Table 16).

**Table 16.** Protocol for determination of total protein content in gill homogenate.

Step	
1	Transfer 10 $\mu\text{L}$ of each protein standards in triplicate to wells on a 96 flat-well multiwell plate
2	Thaw 28 previously homogenized gill samples thoroughly
3	Centrifuge (2200 g; 0.5 min; 4°C)
4	Transfer 10 $\mu\text{L}$ of each sample in triplicate to the assay plate
5	Add 200 $\mu\text{L}$ of a freshly made 50:1 solution of bicinchoninic acid (BCA) and 4% copper II sulphate pentahydrate solution (CuII; 20 mL BCA+400 $\mu\text{L}$ CuII per plate) to each well using a multiway pipette
6	Incubate (37°C, 30 min)
7	Cool to room temperature and measure the absorbance at 560 nm using a multiwell spectrophotometer (equipment described in Table 14, step 11)

**Figure 9.** Typical (a.) ADP and (b.) protein standard curves shown with their corresponding linear regression line and equations as used during Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase. analysis.

### Calculations

The total protein content in 10  $\mu\text{L}$  of gill homogenate was determined (equation 16) and gill  $\text{Na}^+$ ,  $\text{K}^+$ , ATPase was expressed in  $\mu\text{mol ADP} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$  (equation 17).

#### Equation 16

$$\text{Total protein} = \frac{\text{Abs}_{560 \text{ nm}} - \text{Prot} (b)}{\text{Prot} (a)}$$

Where: Total protein is the total protein content in one well ( $\mu\text{g} \cdot 10\mu\text{L}^{-1}$ );  $\text{Abs}_{560 \text{ nm}}$  is the sample absorbance red at 560 nm; Prot (b) is the y-axis intercept of the protein standard curve; and Prot (a) is the regression coefficient of the protein standard curve (Fig. 9b).

#### Equation 17

$$\text{Gill Na}^+\text{K}^+\text{ATPase activity} = \frac{(\text{AMO}_{t_{10-t_2}} - \text{AMI}_{t_{10-t_2}})}{\text{ADP} (a)} * \frac{7.5}{\text{Total protein}}$$

Where: ( $\text{AMO}_{t_{10-t_2}}$ ) is the difference in the sample absorbance at 340 nm between a 10 and 2 min incubation period (8 min interval) when supplemented with the assay medium in presence of the ATPase inhibitor ouabain. Similarly, ( $\text{AMI}_{t_{10-t_2}}$ ) is the difference in sample absorbance at 340 nm between a 10 and 2 min incubation period when supplemented with the assay medium in presence of imidazole buffer. Initial absorbance is taken at 2 min as NADH oxidation stabilized after 2 min following assay medium transfer (McCormick, 1993). The differential absorbance ( $\text{AMO}_{t_{10-t_2}} - \text{AMI}_{t_{10-t_2}}$ ) is divided by the regression coefficient of the ADP standard curve (ADP (a); Fig.9a) to determine the total ATPase activity (in  $\text{nmol ADP} \cdot 10\mu\text{L}^{-1} \cdot 8 \text{ min}^{-1}$ ) in the sample well. This was then multiplied by 7.5 (60 min/8 min) and divided by the total protein content in the sample well (in  $\mu\text{g} \cdot 10\mu\text{L}^{-1}$ ; equation 16) to express Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in  $\text{nmol ADP} \cdot \mu\text{g protein}^{-1} \cdot \text{h}^{-1}$  which is equivalent to  $\mu\text{mol ADP} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ .

## 9. Statistical analysis

The statistical methods used are described in Zar (1999). All statistical analyses were performed using Minitab Statistical software package (Version 15.1, Minitab Inc.; Pennsylvania, USA) or SPSS (Version 15.0, SPSS Inc.; Illinois, USA) unless otherwise stated. A significance level of 5% ( $P < 0.05$ ) was used for all statistical tests.

### 9.1. Basic calculations

#### Arithmetic mean and coefficient of variation

The arithmetic or sample mean ( $\bar{X}$ ) was used to provide an estimate of the population mean ( $\mu$ ) together with the standard error of the mean (SEM) to represent sample distribution, both of which were calculated using Microsoft Excel software.

The coefficient of variation (CV) expresses the variability of the sample relative to its mean (equation 18).

#### Equation 18

$$CV = \left( \frac{\sigma}{\bar{X}} \right) * 100$$

Where: The coefficient of variation (CV) is expressed in %;  $\sigma$  is the standard deviation of the sample population and  $\bar{X}$  is the sample mean.

### 9.2. Regression coefficient and linear correlation analysis

The type of WLR in our populations (*Paper III*) was determined by testing the regression coefficient obtained from least-square regression of logarithmic WLR curve against the isometry (regression coefficient = 3) using a Student t-test (Arslan *et al.*, 2004). The WLR was defined as isometric if its regression coefficient was not significantly different to 3 and positively or negatively allometric if significantly superior or inferior to 3 respectively.

Direction of body size dimorphisms (BW, FL, K; **Paper III**) was determined using log-transformed data by plotting the male or maturing mean body size parameter against the corresponding female or immature values at each sampling point. The reduced major axis (RMA) regression slope was then determined using PAST software (Version 2.02; Paleontological Statistics, University of Oslo, Norway) and tested against the isometry (regression coefficient = 1) using a Student t-test. The RMA regression slopes were never significantly different to 1 indicating a dimorphism proportionally constant in our datasets (Dale *et al.*, 2007).

Linear correlation between two variables (**Paper VI**) were calculated using the Pearson product moment correlation coefficient ( $r$ ). Run's test was used to check for linearity and all linear relationship presented had a slope significantly different to 0 (Instat version 3.0.; GraphPad Software Inc., California, USA).

### 9.3. Chi-square tests

A Chi-square test for goodness of fit was used to determine statistical differences from the expected theoretical 1:1 sex-ratio or from a 50% correct prediction rate of gender or maturity status (see discriminant analysis; **Paper III**). The test was performed with one nominal variable (gender or maturity) and, in each case, two categories (male or female and immature or mature respectively). The null hypothesis tested is that the number of observations in each category is equal to the number of observations theoretically predicted (50% male or 50% correct prediction respectively).

A Chi-square test of independence was used to determine statistical differences in maturation rate between treatments and in the proportion of colour classes between sites (two nominal variables in each case; **Paper III, V and VIII**). Using the observed frequencies, this test calculates the expected frequencies of each category was



calculated then compared between treatments or site using the null hypothesis that the relative proportions of each category did not differ.

#### **9.4. Discriminant analysis (*Paper III*)**

Discriminant analyses were performed with the aim of predicting individual's gender or maturity status using BW, FL and K together or BW alone as predicting factor (SPSS; Kadri *et al.*, 1997a). Analyses were performed using each site's own dataset or using the dataset from another site as a model. Further descriptions are given in the corresponding chapter. Based on Wilks-lambda multivariate test the predicting models were always a good fit for the data ( $P < 0.01$ ).

#### **9.5. Analysis of variance for multiple samples dataset using parametric tests**

##### **Normality testing and homogeneity of variance**

Parametric statistical tests were performed on randomly sampled datasets assuming independence of observations. Normality of the sample distribution was assessed using a Kolmogorov-Smirnov test which compares the distribution of the experimental data with that of an ideal normal (Gaussian) distribution. Homogeneity of variance was tested using the Bartlett's test and/or through examination of the residual plots when the number of samples was large. Datasets sets with a non-normal distribution and/or a heterogeneous variance were transformed (logarithmic, square-root or power transformation) as to meet the assumptions required for parametric testing. In few instances outliers were removed from the dataset; they never exceeded 2% of the total number of samples. Proportions and percentage data were arcsine transformed before analysis.

**Analysis of variance**

Data involving three or more samples were analysed using a one-, two- or three-way analysis of variance (ANOVA) manipulated by General Linear Model (GLM) when appropriate. Replicates were nested within the ANOVA and pooled before further analysis where no significant differences occurred. Dataset where the same fish were repeatedly measured at different time points (*Paper IV*) was analysed using a repeated measure ANOVA. All parameters were considered as fixed effect except family in the triploid study considered as a random affect such that a mixed model ANOVA was used in that case. Where significant differences occurred, Tukey's multiple comparison *post-hoc* test was applied in all significant factor/interaction levels.

Note: Non parametric testing for analysis of categorical variable (Kruskal Wallis Test with Dunn's Multiple Comparison post-hoc) and ANCOVA were performed in one instance (*Paper VIII*).

**CHAPTER 4**  
**BIOMASS MANAGEMENT**

## CHAPTER 4

### PAPER III

#### **RESEARCH ARTICLE**

#### **BODY SIZE DIMORPHISM OF SEA-REARED ATLANTIC SALMON (*SALMO SALAR* L.): IMPLICATIONS FOR THE MANAGEMENT OF SEXUAL MATURATION AND HARVEST QUALITY**

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**Contributions:** The present manuscript was compiled and written in full by the author of this thesis. Sampling, lab and statistical analysis have been carried out by the candidate with the support from the thesis supervisors (Drs. Herve Migaud and John Taylor) who also provided assistance with the experimental design, statistics and proofreading of the manuscript. Support on-site was also provided by Dougie Hunter.

**Keywords:** Atlantic salmon, dimorphism, sexual maturation, prediction, weight-grading, stock management.

**Abstract**

Body size dimorphism between immature and early sexually recruited cohorts of farmed Scottish Atlantic salmon was investigated with the view to optimizing the practical management of early maturation over the second-year at sea. Mixed-sex smolts from a single strain and freshwater source were stocked into four discrete commercial sites and sampled at harvest from June to December 2007, 15 to 22 months post-sea transfer. Individuals were sexed and their maturity status determined based on gonadosomatic-index (GSI) and oocyte leading stage. Whole body weight (BW), fork length (FL) and Fulton condition factor (K) were measured and flesh quality analyzed. The immature mixed-sex population and each gender analyzed separately had an isometric weight-length relationship (WLR) but exhibited seasonal variations in K. Body sizes of immature Atlantic salmon were consistently sexually dimorphic with males exhibiting a higher BW (+13.4%) and FL (+5.9%) but a lower K (-5.0%) than females. Individuals at an early stage of sexual maturation had a significantly higher BW (+35.2%) and K (+20.6%) than the immature cohort in June and July. During this period BW, FL and K together or BW alone were strong and standard indicators of early maturity in our discrete sites. Body size dimorphism described in this study show that sex-ratio is an important parameter of farmed Atlantic salmon populations which is likely to vary following weight-grading and that population composition (sex-ratio and maturation rate) affects the seasonality in K typically observed at harvest. Importantly, the commitment of Atlantic salmon into maturation in spring can be rapidly and accurately estimated in a number of discrete populations by using simple weight-length morphological indicators characterized in a single rearing unit. Following maturation rate estimation, weight-grading implemented according to the predicted stock morphological structure could be used to selectively harvest a high proportion of

maturing individuals at a stage where their flesh quality remains optimal. This could be applied as a powerful and practical on-site maturation management tool in the salmon industry as well as in other commercially important fish species.

## 1. Introduction

Sexually mature Atlantic salmon (*Salmo salar*) have a reduced flesh quality and distinctive skin colouration based on which they are downgraded and lost for human consumption (Michie, 2001). Besides their poor commercial value once harvested, salmonids undergoing maturation can be immunodepressed leading to increased disease susceptibility and mortality rate during on-growing (Bruno, 1989; Salte *et al.*, 1995; Traxler *et al.*, 1997; St-Hilaire *et al.*, 1998; Currie and Woo, 2007). Feeding and growth patterns of maturing Atlantic salmon are also altered being enhanced at an early stage of sexual development (up to summer in Scotland) then reduced during gonadogenesis from late summer onward (Aksnes *et al.*, 1986; Kadri *et al.*, 1997a and b). These successive anabolic and catabolic windows inherent to reproduction are likely to compromise feeding performance of the cohabiting immature cohort due to increased competition and then run the risk of feed-waste from biomass overfeeding. Overall, health, welfare, growth and feeding performance of the whole stock are at risk where significant rates of sexual maturation occur. This is particularly true during the second year at sea when the reproductive window, which extends over the second-half of the year, typically coincides with high water temperature, maximum standing biomass and feeding rate. In order to address this bottleneck, the on-growing salmon industry routinely applies artificial continuous light (LL) from the winter to summer solstice during the second-year at sea. This photoperiod regime is recognized as the most effective (Hansen *et al.*, 1992; Taranger *et al.*, 1998; Endal *et al.*, 2000) and is overall

most successful as a commercial management practice. However, sporadic events of high maturation rates are still experienced, hence the need for a complementary strategy toward the comprehensive prevention of maturation at harvest.

Poorly studied as a potential management tool, the altered growth pattern of sexually recruited individuals is concomitant with body size dimorphism between maturity cohorts. In this respect Kadri *et al.* (1997a) found that, in one sea-winter salmon, relative condition factor and fork-length (FL) but not whole body weight (BW) were good indicators of sexual initiation in advance of gonadogenesis, a period when skin colour and flesh quality remains optimal (Aksnes, 1986, Kadri *et al.*, 1997b). Nonetheless, these predictors were specific to each population/site assessed and did not lead to practical recommendations. Similarly, weight-grading of the stock is routinely performed at sea and is now recognized as playing a part in minimizing maturation rate at harvest, i.e. by segregating best performers for early harvest ahead of their maturation window. However, grading schemes presently applied aim primarily at managing stock variability, density and harvest quality and hence remain empiric and inconsistent for the management of sexual maturity. Furthermore the Scottish salmon industry is nowadays highly concentrated with, in 2007, 93% of the total annual harvest (130,000t) produced by 10 companies, 70% of which was in 55 sea-sites producing over 1,000t a year (Marine Scotland, 2009). While this scale of production does not often permit “visual grilse grading” as traditionally performed, it allows stocking large-scale sites with a single freshwater population but also different sites with populations from the same strain and a close freshwater history. During further on-growing a high number of related but discrete populations within different pens or sites might accordingly exhibit standard size dimorphism between maturity cohorts. If consistent they could, once characterized in a representative rearing unit, be applied to other ones for rapid and non-

invasive estimation of their maturation rates followed by the selective harvest of a high proportion of maturing individuals by simple weight-grading. Such a management strategy would optimize the output of superior quality biomass while isolating the immature cohort from maturing individuals but its feasibility remains poorly assessed.

In association with the dimorphism in growth, differences in Fulton condition factor (K) between maturity cohorts were reported in Atlantic salmon (Aksnes *et al.*, 1986; Rowe *et al.* 1991; Peterson and Harmon, 2005). This parameter is primarily a shape indicator (Froese, 2006) that correlates positively with “visual fatness score”, i.e. rounded appearance, and filleting yield in Atlantic salmon and Atlantic cod (*Gadus morhua*, Einen *et al.*, 1998, 1999; Margeirsson *et al.*, 2007). In this respect K alone can dictate different quality grades at processing and is regularly assessed during on-growing to achieve quality targets. Importantly K is also a recognized indicator of nutritional status in Atlantic salmon where it correlates positively with dietary energy fed (Young *et al.*, 2006) and total flesh lipid at the parr stage (Herbinger and Friars, 1991). In teleosts, the accepted form of weight-length relationships (WLR):  $BW = aFL^b$ , where BW and FL are body size variables and a and b are parameters of the relationship (Froese, 2006), also expresses variations in K over a population size range. Overall constant in populations with an isometric WLR ( $b=3$ ), heavier individuals have a higher K in populations exhibiting a positive allometric relationship ( $b>3$ ) and inversely a smaller K in populations with a negative allometric WLR ( $b<3$ ). With surprisingly few data on Atlantic salmon WLR, this species has like most other teleosts a recognized isometric growth type (Wootton, 1990; Froese and Pauly, 2009) suggesting that a constant shape, K could be achieved for any given length at sea. Nonetheless, a seasonality in K has been reported in farmed stocks that could arise from a seasonal pattern in weight-length development while K appears to be also affected by light-



manipulation (Oppedal *et al.*, 1999, 2003; Johnston *et al.*, 2003; Nordgarden *et al.*, 2003; Young *et al.*, 2006). In the industry, a detrimental spring and early summer drop in K is usually considered as the result of sub-optimal feeding but may also be exacerbated by other management practices.

The main objective of this study was to investigate body size dimorphism between immature and sexually recruited fish in four discrete sea-water populations with the view to optimize the practical management of early maturation during on-growing.

## **2. Materials and methods**

### **2.1. Fish stock and rearing conditions**

Atlantic salmon smolts from the same genetic strain and freshwater origin were transferred to sea as S0+ (underyearling) post-smolts from the 30 January 2006 to the 9 March 2006 with an average weight of  $95 \pm 3\text{g}$  ( $n=2,242,060$ ; 22 sea transfers). Individuals were reared in square pens (24mx24m sides, 10m to 12m depth) in four different commercial sea sites (Sites A, B, C and D) located on the same Scottish loch system (Loch Linnhe: Lat:  $56^{\circ}39'19''$ , Long:  $-5^{\circ}19'56''$ ) up to harvest size. Over the second-year at sea, stocks were fed two commercial diets (Site A, B and C: 35% protein, 32% lipids and 16.5% carbohydrates; Site D: 34% protein, 37% lipids, and 13.5% carbohydrates; Skretting, Invergordon, UK) according to manufacturer recommendations based on monthly sample weight adjusted daily to estimated biomass and observed feeding response (Average daily feeding rate over the production cycles =  $0.71\%$  body weight.day<sup>-1</sup>). Temperature, salinity at 6m depth and water turbidity by means of Secchi disk were measured daily throughout the production cycle. The light-manipulation strategy was common among sites with LL applied using submerged metal-halide lighting units (400W/unit, 4 units/cage, Pisces 400, BGB Engineering,

Grantham, UK) from early January 2007 to mid-June 2007 only in pens to be harvested from August onward, i.e. over the reproduction window. Also consistent among sites was the top-crop harvest strategy consisting of segregating best performing individuals during on-growing and harvesting individual pens with the highest standing BW. This was achieved through biomass weight-grading performed for each pen between October 2006 and February 2007. The harvest season extended from April 2007 to December 2007 in which each site had more than 50% of the biomass harvested between September and December. The total harvested biomass (10,116.5t) was 3708.5t, 3016.6t, 1747.0t and 1644.4t in site A, B, C and D respectively.

## 2.2. Data sampling and analysis

For each site, a cross sectional monthly sampling was performed on harvest batches at the processing plant from June to December 2007. Up to three distinctive skin colour categories were subjectively observed: silver, intermediary (green-back and white belly) and nuptial. Their prevalence was determined by a minimum of 600 observations from at least three counting sessions over the batch-processing period (Data not shown). At each sampling point, a minimum of 25 individuals/sex/skin colour were randomly sexed. Among them, 25 to 30 individuals/sex/skin colour class were measured for BW ( $\pm 0.1$ g), FL ( $\pm 0.1$ cm) and gonad weight (GW) ( $\pm 0.01$ g). K was calculated as  $K = (BW \times 100) / FL^3$  and gonado-somatic-index (GSI) as  $GSI(\%) = (GW \times 100) / BW$ . This sampling strategy allowed optimal characterization of gender and maturity cohorts. Males were classified as immature or sexually recruited based on their bimodal GSI frequency distribution in the population (Kadri *et al.*, 1997a) with a threshold value of  $GSI = 0.20\%$ . Female ovary samples were preserved in Bouin's fixative for 24h before being processed for histological observation and classified

according to their leading oocyte stage (Taranger *et al.*, 1999). They are referred to as immature up to the oil drop stage, as (early) maturing or initiating at the primary and secondary yolk stages indicating the onset of, and commitment toward, sexual maturation and as mature or sexually advanced when the tertiary yolk stage was reached. The left-hand side flesh Scottish quality cut of 10 females/skin colour class/sampling point was stored at -20°C until analysis of flesh colour by means of subjective colour card rating and tristimulus colorimeter, and of total carotenoid and lipid content using near infrared reflectance according to standard industrial practices (Robb, 2001; Fjellanger *et al.*, 2001).

### 2.3. Statistical analysis

The accepted form of WLR in fish:  $BW=aFL^b$ , where BW and FL are body size variables and a and b are parameters of the relationship is presented for illustration and was transformed into its logarithmic equivalent:  $\log(BW)=\log(a)+b*\log(FL)$  for analysis by least-square regression based on individuals pooled per 1cm fork length (Froese, 2006) using Microsoft Excel software. Using b-parameter from WLR analysis at each sampling point, mean- $b\pm SE$  was also determined as this parameter was suggested to better represent the actual WLR of a species (Froese, 2006). It may also be a better indicator of the shape of individuals relative to one another achieved on average in individual harvest batches. Significant variations from the isometry (slope=3) were determined using a Student t-test ( $\alpha=0.05$ ) (Arslan *et al.*, 2004). Harvest batch sex-ratio was calculated by performing a weighted average of gender proportion in each skin colour category. Significant variation of the sex-ratio from 1:1 was determined using a chi-square goodness of fit test. Body size dimorphisms in BW, FL and K between genders at an immature stage and between cohorts of sexual development (both genders

pooled) were calculated at each sampling point and averaged according to Saillant *et al.* (2001). Direction of size dimorphisms (isometry, negative or positive allometry) in datasets was determined using log-transformed data by plotting the male or maturing mean body-size parameter against the corresponding female or immature values at each sampling point and testing the Reduced Major Axis (RMA) regression slope. This was determined using PAST software (Paleontological Statistics Version 2.02; University of Oslo, Norway) against the isometry (slope=1; t-test,  $\alpha=0.05$ ) with a slope not significantly different to 1 indicating a dimorphism proportionally constant among the sampling points (Dale *et al.*; 2007).

The effects of gender or maturity status and time on morphological (BW and K) and flesh quality parameters (total lipid and total carotenoid) were assessed on individual sites by applying a General Linear Model (GLM) with a significance level of 5% using Minitab v.15. Prior to analyses, datasets were checked for normality using the Kolmogorov-Smirnov test and for homogeneity of variance using Bartlett's test. Log or square root transformations were applied when required and proportions were arcsin-transformed. Normality and homogeneity of variance were confirmed by observation of residual plots. Where statistical differences occurred, a Tukey's post-hoc multiple comparisons test was applied (Zar, 1999). Discriminant analyses were performed on June and July samples pooled per site to determine the correct prediction rate of gender or maturity status in each site with either individual's BW, FL and K together or BW alone as predicting parameters using SPSS v.15.0 (modified from Kadri *et al.*, 1997a). Only three sites were analyzed for prediction of maturity status due to the reduced number of individuals initiating sexual development in site D. For prediction of gender and maturity status, analyses were performed using each site's own dataset with the correct prediction rate being provided directly by the software. Prediction of maturity

status was also performed on each site using the two other discrete site datasets to assess if body size dimorphism between maturity cohorts characterized in one site could be used as an indicator of maturation in another discrete site. The predicted maturity status of each individual, output of the discriminant analysis, was then compared to its actual maturity status determined from GSI and histological analysis to establish the rate of correct prediction in each analysis. In a similar fashion, discriminant analyses were also performed to determine the power of GSI in predicting the vitellogenic status of females with a GSI below 1%. Based on Wilks-lambda multivariate test the predicting models were always a good fit for the data ( $P < 0.01$ ).

### **3. Results**

Among the four sites and over the rearing cycle, temperature profiles were identical with an average water temperature of  $10.7 \pm 0.1^\circ\text{C}$ . Average Secchi depth varied from  $4.8 \pm 0.1\text{m}$  (Site A) to  $6.8 \pm 0.1\text{m}$  (Site C) and strong differences in salinity profiles were observed with constant full strength seawater in sites C and D (33.9ppm to 34.9ppm) and variable salinity in sites A and B ( $20.1 \pm 0.3\text{ppm}$  and  $27.0 \pm 0.2\text{ppm}$  respectively).

#### **3.1. Immature population**

##### **Weight-Length Relationship (WLR) analysis and population sex-ratio**

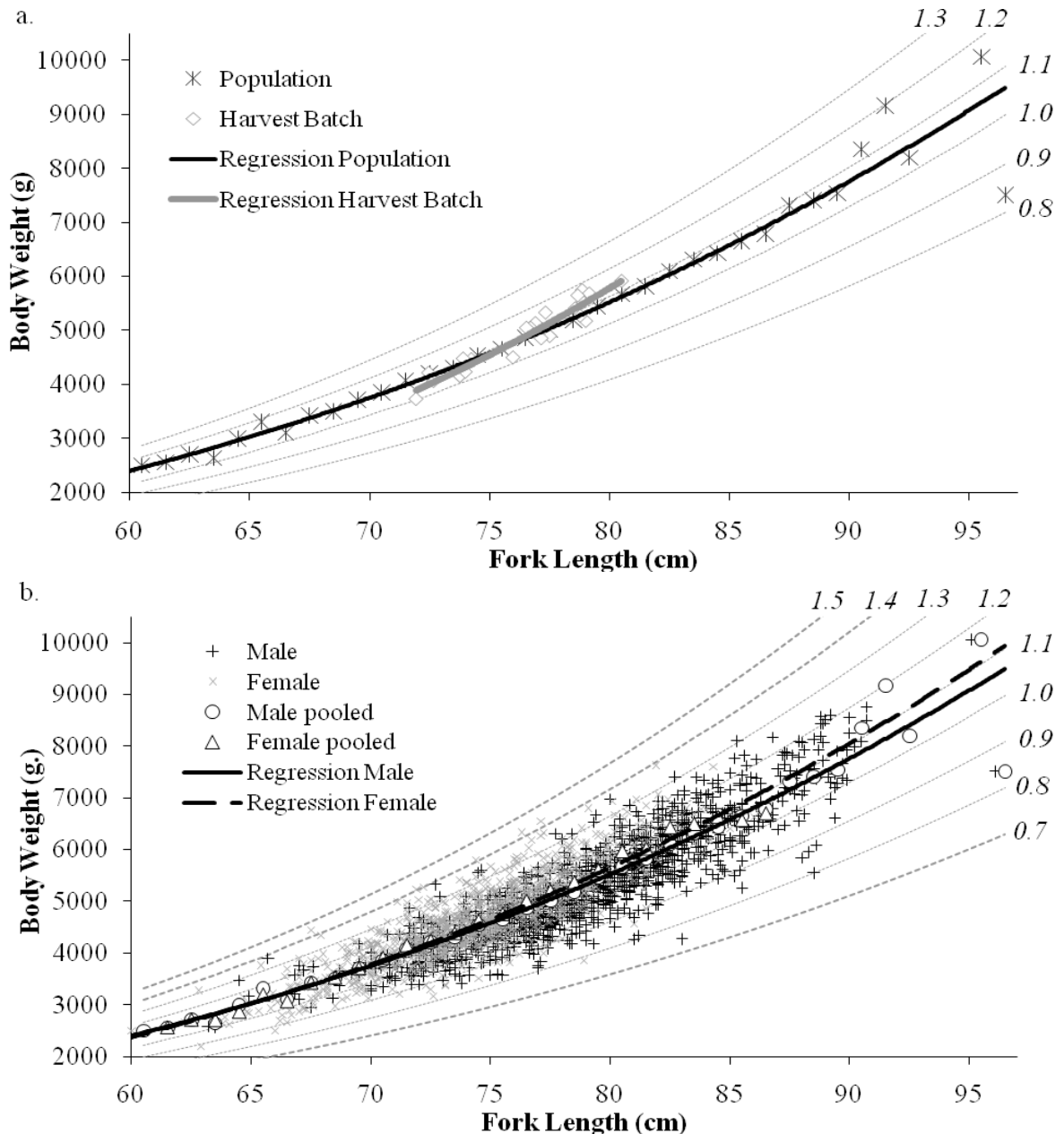
Over the sampling period the sex-ratio significantly varied from the expected balanced 1:1 ratio in 13 out of the 23 sampling points assessed. From June to August inclusive the proportion of males was significantly higher than the proportion of females in 6 out of 11 harvest batches sampled whilst females never were. Conversely in harvest batches from September onward ( $n=12$ ), females were dominant in 5 harvest

batches and males only in 2 (Table 1a). Morphological parameters of the immature cohort sampled over Xa 7-month period in the four sites were as follows:  $n=2235$ ,  $BW=5013\pm 22g$  ( $p_5-p_{95}=3528g-6860g$ ),  $FL=76.9\pm 0.1cm$  ( $p_5-p_{95}=69.3-85.5cm$ ),  $K=1.09\pm 0.02$  ( $p_5-p_{95}=0.93-1.26$ ) where p values indicate the percentiles.

**Table 1** (a.) Number of individuals sexed and proportion of males within each harvest batch for each of the four sites studied. Significant difference to the expected balanced sex-ratio (1:1) was determined by a chi-square test for goodness of fit ( $\alpha=0.05$ ) and shown in bold when significantly skewed toward an excess of Male (M; +) or Female (F; -). (b.) Average whole body weight (BW) and (c.) Fulton condition factor (K) of immature males and immature females within each harvest batch for each of the four sites studied. Values are expressed as mean $\pm$ SE with  $n=30$  fish/sex/month/site. Significant differences between genders within each harvest batch are shown in bold. Significant differences between monthswithin each site and gender cohort are shown by different superscripts (GLM,  $\alpha=0.05$ ).

Site		June	July	August	September	October	November	December
<b>(a.) N Ind. (% Male)</b>								
<b>A</b>		<b>106 (71.7+)</b>	197 (49.3)	161 (53.9)	<b>235 (62.5+)</b>	<b>187 (41.8-)</b>	<b>242 (63.3+)</b>	239 (48.4)
<b>B</b>		62 (51.6)	<b>145 (58.9+)</b>	<b>163 (62.6+)</b>	<b>187 (39.2-)</b>	169 (46.2)	175 (42.6)	173 (56.0)
<b>C</b>			<b>168 (63.6+)</b>	<b>194 (58.0+)</b>		187 (44.4)	<b>226 (38.4-)</b>	
<b>D</b>		63 (52.4)	122 (49.4)	<b>136 (60.1+)</b>	<b>113 (37.9-)</b>		<b>211 (38.4-)</b>	
<b>(b.) BW (g)</b>								
<b>A</b>	M	<b>4498<math>\pm</math>169<sup>a</sup></b>	4399 $\pm$ 120 <sup>a</sup>	4603 $\pm$ 77 <sup>a</sup>	<b>5928<math>\pm</math>116<sup>cd</sup></b>	<b>5437<math>\pm</math>151<sup>bc</sup></b>	<b>5368<math>\pm</math>130<sup>b</sup></b>	<b>6196<math>\pm</math>134<sup>d</sup></b>
	F	<b>3644<math>\pm</math>91<sup>a</sup></b>	3934 $\pm$ 106 <sup>ab</sup>	4340 $\pm$ 84 <sup>b</sup>	<b>5115<math>\pm</math>86<sup>c</sup></b>	<b>4834<math>\pm</math>101<sup>bc</sup></b>	<b>4429<math>\pm</math>92<sup>b</sup></b>	<b>5289<math>\pm</math>104<sup>c</sup></b>
<b>B</b>	M	4446 $\pm$ 97 <sup>a</sup>	4365 $\pm$ 99 <sup>a</sup>	5283 $\pm$ 92 <sup>b</sup>	<b>6202<math>\pm</math>91<sup>c</sup></b>	<b>6313<math>\pm</math>184<sup>c</sup></b>	5633 $\pm$ 164 <sup>bc</sup>	5971 $\pm$ 185 <sup>c</sup>
	F	4001 $\pm$ 77 <sup>a</sup>	4116 $\pm$ 67 <sup>a</sup>	4817 $\pm$ 68 <sup>b</sup>	<b>5156<math>\pm</math>99<sup>bc</sup></b>	<b>5509<math>\pm</math>119<sup>c</sup></b>	5027 $\pm$ 116 <sup>bc</sup>	5318 $\pm$ 110 <sup>bc</sup>
<b>C</b>	M		<b>4883<math>\pm</math>108<sup>a</sup></b>	5452 $\pm$ 114 <sup>ab</sup>		<b>5791<math>\pm</math>157<sup>b</sup></b>	<b>5620<math>\pm</math>149<sup>b</sup></b>	
	F		<b>3934<math>\pm</math>106<sup>a</sup></b>	4882 $\pm$ 91 <sup>b</sup>		<b>5023<math>\pm</math>113<sup>b</sup></b>	<b>4918<math>\pm</math>125<sup>b</sup></b>	
<b>D</b>	M	<b>4035<math>\pm</math>89<sup>a</sup></b>	<b>4797<math>\pm</math>80<sup>bc</sup></b>	<b>5156<math>\pm</math>96<sup>c</sup></b>	4645 $\pm$ 117 <sup>b</sup>		<b>5315<math>\pm</math>138<sup>c</sup></b>	
	F	<b>3446<math>\pm</math>97<sup>a</sup></b>	<b>4214<math>\pm</math>60<sup>b</sup></b>	<b>4557<math>\pm</math>89<sup>bc</sup></b>	4325 $\pm$ 98 <sup>b</sup>		<b>4785<math>\pm</math>108<sup>c</sup></b>	
<b>(c.) K</b>								
<b>A</b>	M	1.04 $\pm$ 0.01 <sup>ab</sup>	1.01 $\pm$ 0.01 <sup>a</sup>	<b>1.05<math>\pm</math>0.01<sup>ab</sup></b>	<b>1.08<math>\pm</math>0.01<sup>bc</sup></b>	<b>1.09<math>\pm</math>0.01<sup>bc</sup></b>	<b>1.02<math>\pm</math>0.01<sup>a</sup></b>	<b>1.13<math>\pm</math>0.01<sup>c</sup></b>
	F	1.06 $\pm$ 0.01 <sup>ab</sup>	1.05 $\pm$ 0.01 <sup>a</sup>	<b>1.14<math>\pm</math>0.01<sup>c</sup></b>	<b>1.14<math>\pm</math>0.01<sup>c</sup></b>	<b>1.15<math>\pm</math>0.01<sup>c</sup></b>	<b>1.07<math>\pm</math>0.01<sup>a</sup></b>	<b>1.19<math>\pm</math>0.01<sup>c</sup></b>
<b>B</b>	M	1.08 $\pm$ 0.02 <sup>b</sup>	<b>1.00<math>\pm</math>0.01<sup>a</sup></b>	<b>1.08<math>\pm</math>0.01<sup>b</sup></b>	1.12 $\pm$ 0.01 <sup>b</sup>	<b>1.09<math>\pm</math>0.01<sup>b</sup></b>	<b>1.10<math>\pm</math>0.02<sup>b</sup></b>	<b>1.11<math>\pm</math>0.02<sup>b</sup></b>
	F	1.14 $\pm$ 0.01 <sup>ab</sup>	<b>1.09<math>\pm</math>0.01<sup>a</sup></b>	<b>1.15<math>\pm</math>0.01<sup>ab</sup></b>	1.15 $\pm$ 0.01 <sup>ab</sup>	<b>1.15<math>\pm</math>0.01<sup>ab</sup></b>	<b>1.20<math>\pm</math>0.01<sup>b</sup></b>	<b>1.19<math>\pm</math>0.02<sup>b</sup></b>
<b>C</b>	M		1.02 $\pm$ 0.01 <sup>a</sup>	1.03 $\pm$ 0.01 <sup>a</sup>		1.08 $\pm$ 0.01 <sup>b</sup>	<b>1.04<math>\pm</math>0.01<sup>ab</sup></b>	
	F		1.05 $\pm$ 0.01 <sup>a</sup>	1.07 $\pm$ 0.01 <sup>a</sup>		1.12 $\pm$ 0.01 <sup>b</sup>	<b>1.10<math>\pm</math>0.02<sup>ab</sup></b>	
<b>D</b>	M	0.99 $\pm$ 0.01 <sup>a</sup>	1.00 $\pm$ 0.01 <sup>a</sup>	1.04 $\pm$ 0.01 <sup>ab</sup>	1.08 $\pm$ 0.01 <sup>bc</sup>		1.10 $\pm$ 0.01 <sup>c</sup>	
	F	1.01 $\pm$ 0.02 <sup>a</sup>	1.05 $\pm$ 0.01 <sup>a</sup>	1.06 $\pm$ 0.01 <sup>a</sup>	1.12 $\pm$ 0.01 <sup>b</sup>		1.14 $\pm$ 0.01 <sup>b</sup>	

The WLRs of the mixed-sex population as a whole and of each gender analyzed separately were isometric over the study period (Fig.1a and 1b with parameters of the WLRs). A strong positive allometric relationship was nonetheless observed when plotting average BW and FL of individual harvest batches (Fig.1a). This was consistent with the clear tendency toward an increase of both BW and K over the harvest season in the immature male, female and mixed-sex cohorts within each site. There was indeed a significant effect of time on BW and K of the immature cohorts in all sites (GLM,  $P < 0.001$ ; Table 1b, 1c, 2a and 2b). Both parameters were significantly lower in June and July. Mean b-parameters for each individual site or all sites pooled were always significantly lower than 3 except in site D (all sites= $2.70 \pm 0.04$ ; site A= $2.70 \pm 0.09$ , B= $2.70 \pm 0.10$ , C= $2.67 \pm 0.09$  and D= $2.76 \pm 0.11$ ) but never significantly different to the isometry when considering genders separately (Male: all site= $2.86 \pm 0.05$ ; site A= $2.84 \pm 0.11$ , B= $2.86 \pm 0.09$ , C= $2.93 \pm 0.11$  and D= $2.79 \pm 0.09$ ; Female: all sites= $2.88 \pm 0.07$ ; site A= $2.94 \pm 0.09$ , B= $2.79 \pm 0.20$ , C= $2.81 \pm 0.13$ , D= $2.97 \pm 0.23$ ).



**Figure 1. (a.)** WLR and least-square regression line of immature population based on individuals from all sampling points (4 sites, 7 months) pooled per 1cm length class ( $y=0.0172x^{2.893}$ , CI-b=2.76-3.02,  $r^2=0.984$ ,  $n=36$ , isometric relationship with P-value=0.105) and using mean-FL and mean-BW at each sampling point (genders average) ( $y=0.0005x^{3.7268}$ , CI-b=3.23-4.22,  $r^2=0.921$ , positive allometry with P-value=0.006). The dashed grey lines represent isometrics WLRs of given condition factor (italic). **(b.)** WLR of the immature females (N=1104) and immature males (N=1131) cohorts with regression line based on individual pooled per 1cm length class (Males:  $y=0.0229x^{2.827}$ , CI-b=2.62-3.03,  $r^2=0.962$ , isometric relationship with P-value=0.097; Females:  $y=0.0104x^{3.0135}$ , CI-b=2.904-3.123,  $r^2=0.993$ , isometric relationship with P-value=0.801).

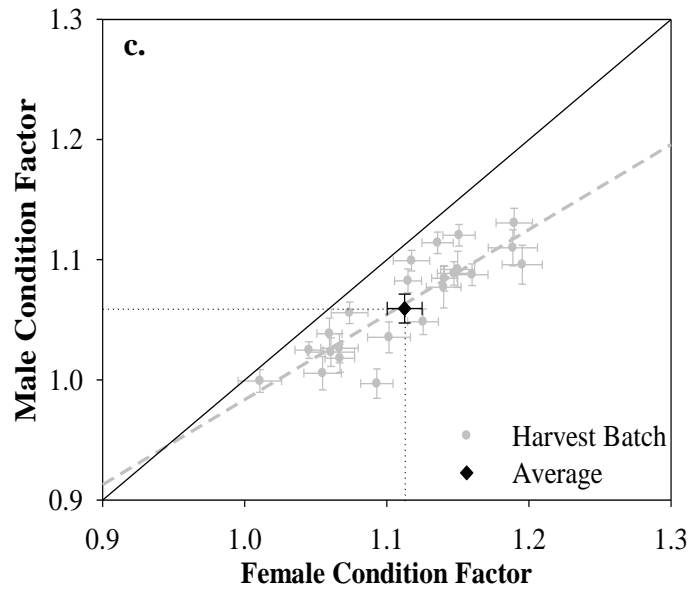
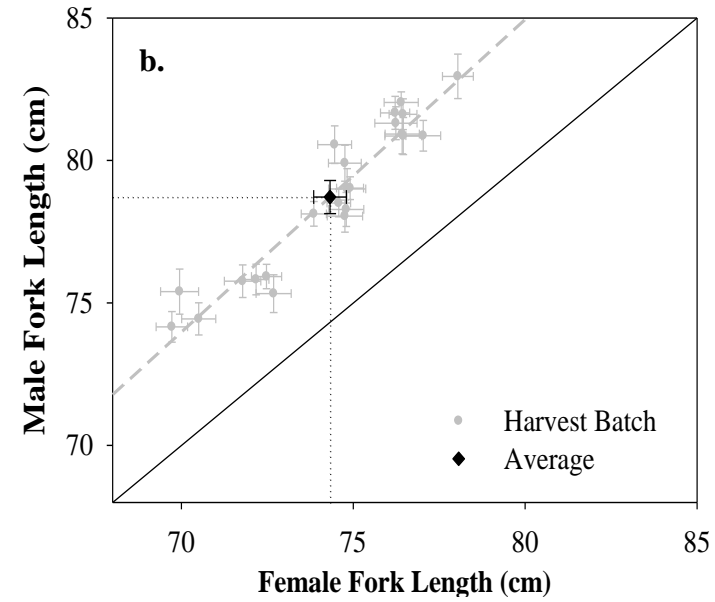
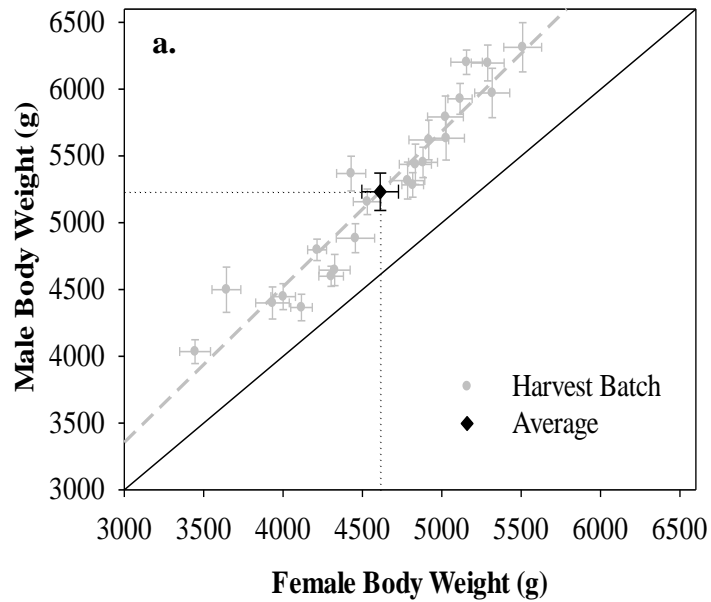


### Sexual size dimorphism (SSD) and discriminant analysis

There was an overall significant effect of sex on BW, K and FL of the immature cohort in each individual site (GLM,  $P < 0.001$ ) with males showing a significantly higher BW in 14 out of 23 harvest batches and a significantly lower K in 11 out of 23 harvest batches (Table 1b and 1c). Sexual size dimorphisms were observed at each sampling point (Fig.2a,b,c) and were proportionally constant as shown by the RMA regression slopes which were never significantly different to the isometry (t-test,  $\alpha = 0.05$ ,  $P = 0.4899$ ;  $P = 0.2630$ ;  $P = 0.1253$  for respectively BW, FL and K). Based on sampling point means ( $n = 23$ ), immature males ( $n = 1131$ ) compared to females ( $n = 1104$ ) were 13.4% heavier ( $BW_{\text{Male}} = 5232 \pm 140\text{g}$ ;  $BW_{\text{Female}} = 4592 \pm 119\text{g}$ ) and 6.1% longer ( $FL_{\text{Male}} = 78.9 \pm 0.6\text{cm}$ ;  $FL_{\text{Female}} = 74.3 \pm 0.5\text{cm}$ ) while females had a 5.0% higher K ( $K_{\text{Male}} = 1.06 \pm 0.01$ ;  $K_{\text{Female}} = 1.11 \pm 0.01$ ). From discriminant analyses using as predictors BW, FL and K of individuals from the site under analysis, 75.8% (Site C) to 78.9% (Site B) of the site's population were correctly classified within their gender cohort. These rates of correct prediction were in all cases significantly higher than 50% which could have been achieved by chance alone. Using BW as a sole predictive factor, correct prediction of gender ranged from 57% (Site C) to 68% (Site D), significantly more than 50% in all but site C (Table 3).

**Table 2.** (a.) Average whole body weight (BW), (b.) Fulton condition factor (K), (c.) total lipid and (d.) total carotenoid of immature (Immat.) and sexually maturing (Mat.) fish within each harvest batch for each of the four sites studied. Values are expressed as mean  $\pm$  SE with (a.) and (b.)  $n = 50-60$  fish/maturity cohort/month/site (except in June for all sites and site D throughout where  $n = 10-15$  maturing fish/site/month) (c.) and (d.)  $n = 10$  female/site/month/maturity cohort. Significant differences between maturity cohorts within each harvest batch are shown in bold. Significant differences between month within each maturity cohort are shown by different superscript letters (GLM,  $\alpha = 0.05$ ). Maturing fish in June and July only were at an early stage of development and are underlined.

Site		June	July	August	September	October	November	December
<b>(a) BW (g)</b>								
<b>A</b>	I.	4086±113 <sup>a</sup>	<b>4139±84<sup>a</sup></b>	4480±58 <sup>ab</sup>	<b>5535±82<sup>de</sup></b>	<b>5127±94<sup>cd</sup></b>	4919±92 <sup>bc</sup>	<b>5802±98<sup>e</sup></b>
	M.	<u>5191±264<sup>abcd</sup></u>	<u>5534±177<sup>a</sup></u>	4099±218 <sup>cd</sup>	<b>4745±142<sup>bc</sup></b>	<b>3883±149<sup>de</sup></b>	5013±154 <sup>ab</sup>	<b>3271±86<sup>e</sup></b>
<b>B</b>	I.	4223±68 <sup>a</sup>	<b>4232±60<sup>a</sup></b>	<b>5054±61<sup>b</sup></b>	<b>5702±83<sup>cd</sup></b>	<b>5890±113<sup>d</sup></b>	<b>5268±100<sup>bc</sup></b>	<b>5684±119<sup>cd</sup></b>
	M.	<u>5610±530<sup>ab</sup></u>	<u>5944±194<sup>a</sup></u>	<b>6186±267<sup>a</sup></b>	<b>4145±206<sup>b</sup></b>	<b>4304±199<sup>b</sup></b>	<b>3812±145<sup>b</sup></b>	<b>3873±139<sup>b</sup></b>
<b>C</b>	I.		<b>4409±93<sup>a</sup></b>	5202±81 <sup>b</sup>		<b>5411±103<sup>b</sup></b>	<b>5282±103<sup>b</sup></b>	
	M.		<u>5543±168<sup>a</sup></u>	5499±174 <sup>a</sup>		<b>4159±158<sup>b</sup></b>	<b>4109±116<sup>b</sup></b>	
<b>D</b>	I.	<b>3730±77<sup>a</sup></b>	<b>4498±57<sup>b</sup></b>	4886±4 <sup>c</sup>	4468±76 <sup>b</sup>		<b>5080±94<sup>c</sup></b>	
	M.	<u>5594±617<sup>a</sup></u>	<u>7025±275<sup>a</sup></u>		3410±269 <sup>b</sup>		<b>3844±100<sup>b</sup></b>	
<b>(b) K</b>								
<b>A</b>	I.	<b>1.05±0.01<sup>ab</sup></b>	<b>1.03±0.01<sup>a</sup></b>	1.09±0.01 <sup>b</sup>	<b>1.11±0.01<sup>bc</sup></b>	<b>1.12±0.01<sup>bcd</sup></b>	<b>1.04±0.01<sup>a</sup></b>	<b>1.16±0.01<sup>d</sup></b>
	M.	<u>1.25±0.07<sup>ab</sup></u>	<u>1.23±0.02<sup>a</sup></u>	1.08±0.03 <sup>b</sup>	<b>1.05±0.01<sup>bc</sup></b>	<b>1.03±0.02<sup>bcd</sup></b>	<b>0.98±0.01<sup>d</sup></b>	<b>1.06±0.02<sup>bc</sup></b>
<b>B</b>	I.	<b>1.11±0.01<sup>b</sup></b>	<b>1.05±0.01<sup>a</sup></b>	1.12±0.01 <sup>b</sup>	1.13±0.01 <sup>b</sup>	1.12±0.01 <sup>b</sup>	<b>1.16±0.01<sup>b</sup></b>	<b>1.14±0.01<sup>b</sup></b>
	M.	<u>1.39±0.04<sup>a</sup></u>	<u>1.28±0.01<sup>a</sup></u>	1.11±0.01 <sup>b</sup>	1.07±0.02 <sup>b</sup>	1.09±0.02 <sup>b</sup>	<b>1.08±0.02<sup>b</sup></b>	<b>1.09±0.02<sup>b</sup></b>
<b>C</b>	I.		<b>1.04±0.01<sup>a</sup></b>	<b>1.04±0.01<sup>a</sup></b>		1.10±0.01 <sup>b</sup>	1.07±0.01 <sup>ab</sup>	
	M.		<u>1.26±0.01<sup>a</sup></u>	<b>1.17±0.02<sup>b</sup></b>		1.07±0.01 <sup>c</sup>	1.06±0.02 <sup>c</sup>	
<b>D</b>	I.	<b>1.00±0.01<sup>a</sup></b>	<b>1.02±0.01<sup>ab</sup></b>	1.05±0.01 <sup>b</sup>	1.10±0.01 <sup>c</sup>		1.11±0.01 <sup>c</sup>	
	M.	<u>1.29±0.03</u>	<u>1.25±0.02</u>		1.05±0.07		1.11±0.02	
<b>(c) Total Lipid (g.100g<sup>-1</sup> flesh)</b>								
<b>A</b>	I.	16.1±0.8	16.6±0.8	<b>16.1±0.5</b>	<b>15.4±0.4</b>	<b>16.6±0.5</b>	<b>15.7±1.0</b>	<b>16.2±0.7</b>
	M.		<u>17.9±0.6<sup>a</sup></u>	<b>10.3±6.3<sup>b</sup></b>	<b>11.0±0.8<sup>b</sup></b>	<b>10.0±0.8<sup>b</sup></b>	<b>10.0±1.2<sup>b</sup></b>	<b>7.8±0.6<sup>b</sup></b>
<b>B</b>	I.	15.1±0.7 <sup>ab</sup>	12.1±0.5 <sup>a</sup>	<b>14.4±0.5<sup>ab</sup></b>	<b>15.8±0.9<sup>b</sup></b>	<b>15.4±0.9<sup>ab</sup></b>	<b>15.3±0.9<sup>ab</sup></b>	<b>15.4±0.8<sup>ab</sup></b>
	M.		<u>14.5±0.8<sup>a</sup></u>	<b>10.1±0.5<sup>b</sup></b>	<b>6.9±1.2<sup>b</sup></b>	<b>7.8±0.9<sup>b</sup></b>	<b>6.8±0.6<sup>b</sup></b>	<b>7.4±0.5<sup>b</sup></b>
<b>C</b>	I.		12.0±0.9 <sup>a</sup>	16.1±0.6 <sup>b</sup>		<b>15.4±0.7<sup>ab</sup></b>	<b>15.9±1.0<sup>b</sup></b>	
	M.		<u>15.3±0.9<sup>a</sup></u>	13.3±0.6 <sup>a</sup>		<b>7.2±0.7<sup>b</sup></b>	<b>8.0±0.9<sup>b</sup></b>	
<b>D</b>	I.	14.7±0.9 <sup>ab</sup>	11.6±0.4 <sup>a</sup>	14.2±0.6 <sup>ab</sup>	15.1±0.7 <sup>b</sup>		<b>14.5±1.1<sup>ab</sup></b>	
	M.		<u>14.3±0.5<sup>a</sup></u>	10.8±1.3 <sup>a</sup>	12.2±1.0 <sup>a</sup>		<b>6.9±0.7<sup>b</sup></b>	
<b>(d) Total Carotenoid (mg.kg<sup>-1</sup> flesh)</b>								
<b>A</b>	I.	6.6±0.2 <sup>a</sup>	7.0±0.2 <sup>abc</sup>	7.6±0.3 <sup>bc</sup>	7.8±0.2 <sup>c</sup>	<b>7.7±0.2<sup>c</sup></b>	<b>7.3±0.7<sup>abc</sup></b>	<b>6.6±0.3<sup>ab</sup></b>
	M.		<u>6.8±0.2<sup>a</sup></u>	6.33±0.6 <sup>ab</sup>	6.6±0.4 <sup>ab</sup>	<b>5.5±0.5<sup>ab</sup></b>	<b>5.1±0.5<sup>b</sup></b>	<b>2.3±0.4<sup>c</sup></b>
<b>B</b>	I.	7.3±0.3 <sup>ab</sup>	7.4±0.2 <sup>ab</sup>	7.6±0.2 <sup>ab</sup>	<b>8.0±0.2<sup>a</sup></b>	<b>7.4±0.2<sup>ab</sup></b>	<b>7.0±0.2<sup>ab</sup></b>	<b>6.9±0.2<sup>b</sup></b>
	M.		<u>8.2±0.2<sup>a</sup></u>	7.3±0.3 <sup>ab</sup>	<b>6.5±0.3<sup>bc</sup></b>	<b>5.9±0.4<sup>c</sup></b>	<b>4.2±0.3<sup>d</sup></b>	<b>2.4±0.2<sup>e</sup></b>
<b>C</b>	I.		7.1±0.3	8.0±0.2		<b>6.9±0.1</b>	<b>7.0±0.3</b>	
	M.		<u>7.3±0.3<sup>a</sup></u>	7.7±0.2 <sup>a</sup>		<b>5.0±0.4<sup>b</sup></b>	<b>3.6±0.3<sup>c</sup></b>	
<b>D</b>	I.	7.9±0.2	7.5±0.2	7.9±0.1	7.7±0.1		<b>7.3±0.2</b>	
	M.		<u>7.8±0.3<sup>a</sup></u>	7.2±0.1 <sup>a</sup>	7.3±0.2 <sup>a</sup>		<b>4.9±0.3<sup>b</sup></b>	



**Figure 2.** Relationship at each sampling point (grey dots) between mean immature males and mean immature females (a.) BW (b.) FL and (c.) K given with their RMA regression line (long spotted grey line). The plain black line is the isometry of equality between genders, the black dot and short spotted black line indicates the harvest batches average. Coefficients of the RMA regression slope were never significantly different to the isometry (a.)  $a=1.05\pm 0.07$ ;  $CI-a=0.92-1.26$ ;  $r^2=0.955$ ; (b.)  $a=1.08\pm 0.07$ ;  $CI-a=0.95-1.29$ ;  $r^2=0.951$ ; (c.)  $0.85\pm 0.09$ ;  $CI-a=0.70-1.06$ ;  $r^2=0.867$ .

**Table 3.** Proportion of correct classification of individuals into their gender cohort based on each site own dataset, June and July sampled pooled, using as predictors whole body weight (BW), fork length (FL) and Fulton condition factor (K) or BW alone. Significant differences from 50% of correct prediction, which could have been expected by chance alone, were determined by using a chi-square test for goodness of fit ( $\alpha = 0.05$ ) and are shown in bold.

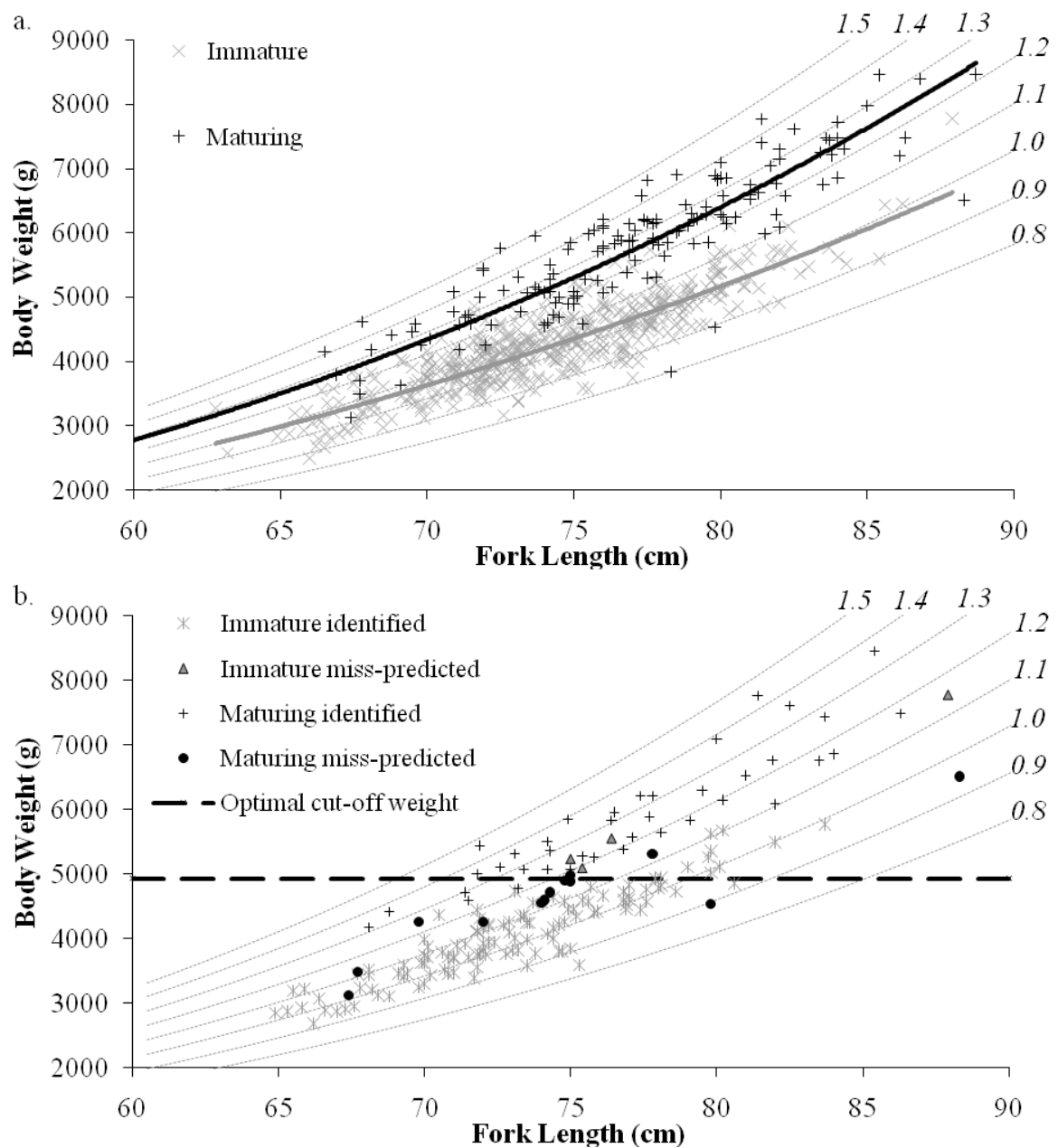
Site	n Ind.	Predictors: BW, FL and K			Predictor: BW		
		Correct prediction (%)	$\chi^2$	P-value	Correct prediction (%)	$\chi^2$	P-value
A	177	<b>77.4</b>	30.03	0.000	<b>64.4</b>	8.29	0.004
B	180	<b>78.9</b>	33.40	0.000	<b>63.9</b>	7.73	0.005
C	119	<b>74.8</b>	24.60	0.000	57.1	2.02	0.156
D	176	<b>75.6</b>	26.21	0.000	<b>68.2</b>	13.25	0.000

### 3.2. Dimorphism between maturity cohorts

#### Dimorphism over the study period

Fish undergoing sexual maturation in June and July were always at an early stage of sexual development based on GSI and oocyte leading stage ( $GSI_{\text{Male}}=0.51\pm 0.02\%$ ;  $GSI_{\text{Female}}=1.37\pm 0.11\%$  at the primary or secondary yolk stage). During this period, a total of 502 immature (Male=244; female=258) and 150 sexually recruited (Male=78; Female=72) individuals were sampled from a total of seven harvest batches and four sites. At each sampling point, maturing fish were 25.7% to 56.1% heavier (significant differences observed in 5 out of 7 harvest batches) than immature siblings and displayed a significantly higher K in all harvest batches (19.0% to 29.0%) (Table 2a and 2b). No differences in FL between maturity cohorts were observed. Based on sampling point means, individuals that initiated sexual development were 37.9% heavier ( $BW_{\text{Recruited}}=5777\pm 224\text{g}$ ;  $BW_{\text{Immature}}=4188\pm 94\text{g}$ ), 4.0% longer ( $FL_{\text{Recruited}}=76.7\pm 1.0\text{cm}$ ,  $FL_{\text{Immature}}=73.8\pm 0.6\text{cm}$ ) and had a 22.6% higher K ( $K_{\text{Recruited}}=1.28\pm 0.02$ ;  $K_{\text{Immature}}=1.04\pm 0.01$ ). Body size dimorphisms between maturity cohorts in June and July are illustrated by the WLR of the 652 individuals sampled over

this period and classified according to their maturity status (Fig.3a). As the maturation cycle progressed, maturing fish lost their body size advantage. They remained significantly heavier in August in site B only and had a higher K in site C. From September onward, maturing fish were at an advanced stage of sexual maturation ( $GSI_{\text{Male}}=3.67\pm 0.06\%$ ;  $GSI_{\text{Female}}=20.30\pm 0.28\%$  always at the tertiary yolk stage) and significantly lighter than immature fish (in 10 out of 12 harvest batches) with also a significantly lower K (in 6 out of 12 harvest batches; Table 2a and 2b). With regard to flesh quality, no significant differences between maturity cohorts were observed in July for subjective colour rating, tristimulus colour composition (data not shown), total carotenoid and total lipid (Table 2c and 2d, GLM,  $P>0.05$ ). The effects of sexual maturation on flesh quality were then detected from August onward with a significantly lower total flesh lipid, measured in two sites in August, followed by a significantly lower total flesh carotenoid observed in one site in September. From October onward, both those parameters were significantly reduced in fish reaching full maturity. Further analyses were therefore performed on the June and July period only that corresponds to the anabolic window of early sexual development prior to flesh quality deterioration as confirmed above.



**Figure 3.** (a.) WLR of immature ( $n=502$ ) and recruited ( $n=150$ ) individuals sampled in June and July, all sites pooled, with their respective least-square regression line. (b.) WLR of immature ( $n=124$ ) and recruited ( $n=53$ ) individuals sampled from site A in June and July and presented as result of the discriminant analysis using a discrete site dataset (Site B) with BW, FL and K as predictors. For comparison, the black dashed line represents the optimal segregating BW between maturity cohorts using BW as sole predictor. Any individuals above the BW threshold were predicted as maturing and any individuals below were predicted as immature. The dashed grey lines represent isometric WLRs of given condition factor (italic).

**Discriminant analysis between immature and early recruited cohorts**

Over the June-July period, discriminant analyses were performed to predict individual maturity status based on their body size parameters. Results are presented in Table 4 and illustrated by a specific example (Fig.3b). When performed on each individual site using its own data set and three external morphological predictors (BW, FL and K), correct prediction of maturity status averaged  $92.2\pm 1.3\%$  of the sites' population and  $87.6\pm 4.1\%$  of their recruited cohort (Table 4a). Using the discriminant functions from different discrete sites, on average  $89.5\pm 1.2\%$  of each site population was correctly classified including  $85.7\pm 4.5\%$  of maturing individuals. In those last analysis,  $81.9\pm 4.0\%$  of individuals predicted as maturing were truly maturing while  $5.9\pm 1.7\%$  of individuals predicted as immature were in fact maturing as determined from GSI and histological analysis. Compared to a true maturation rate averaging  $31.2\pm 4.0\%$  in our four site datasets, the predicted maturation rate averaged  $33.2\pm 3.9\%$  with an error on individual sites of  $-5.1\%$  (Site A)  $+6.1\%$  (Site B) and  $+5.0\%$  (Site C).

Analyses were also performed using BW as the sole discriminating factor between maturity cohorts allowing determination of an optimal BW threshold between maturity cohorts (Table 4b). Using each site's own dataset the maturity status of on average  $84.1\pm 1.6\%$  of the populations and  $76.5\pm 2.0\%$  of the recruited cohorts was correctly predicted. Using discrete site datasets the maturity status of on average  $80.1\pm 2.5\%$  of the populations and  $71.8\pm 7.1\%$  of the recruited cohorts was correctly classified.

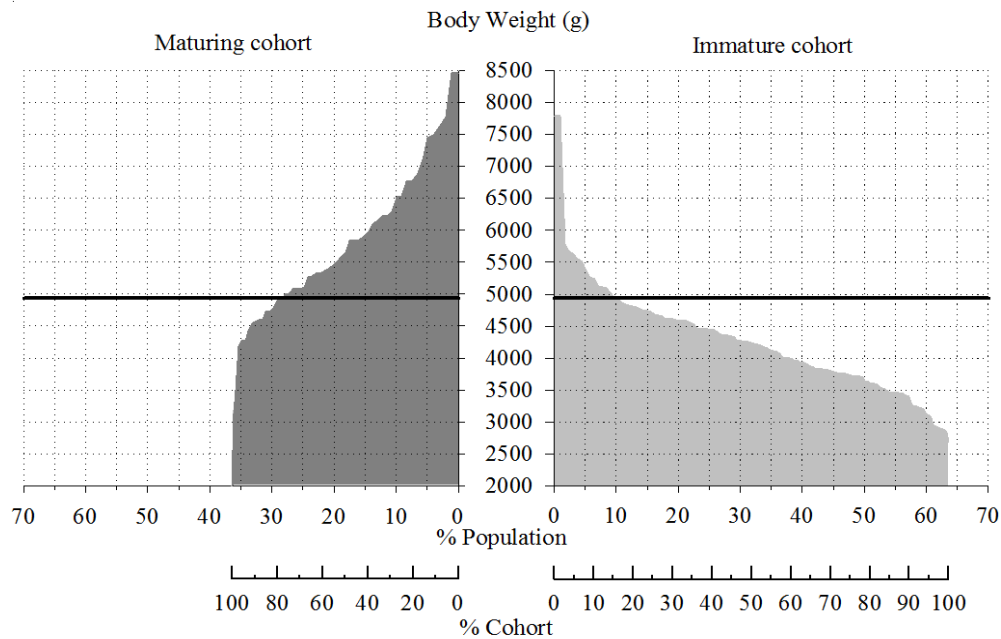
Compared to the analyses based on three morphological predictors, using BW alone reduced the accuracy of the prediction in the different site populations by an average of  $9.4\%$  (based on site's own dataset) and  $13.9\%$  (based on discrete site datasets). Furthermore, an average of  $34.7\pm 7.7\%$  of the populations, including  $71.8\pm 7.1\%$  of truly recruited fish, were over the optimal BW threshold between

**Table 4** Results (mean  $\pm$  SE) from analysis of discriminant between maturity cohort performed on site A, B and C on June and July datasets pooled (except site C where no harvest occurred in June) using (a.) BW, FL and K as predictors and (b.) BW as sole predictor. For each set of predictor used, analyses were done using the site own dataset and the 2 discrete site datasets for which the true composition of the cohorts predicted as immature and maturing is presented.

	Site	A	B	C	Average
<b>Initial population</b>					
Immature (n)		124	135	73	111 $\pm$ 19
Maturing (n)		53	45	46	48 $\pm$ 3
Maturation rate (%)		29.9	25.0	38.7	31.2 $\pm$ 4.0
<b>(a.) PREDICTORS: BW, FL, K</b>					
• <b>Correct prediction from own site dataset (%)</b>					
Immature		91.9	96.3	93.2	93.8 $\pm$ 1.3
Maturing		84.9	82.2	95.7	87.6 $\pm$ 4.1
Population		89.8	92.8	94.1	92.2 $\pm$ 1.3
• <b>Correct prediction from discrete site datasets (%)</b>					
Immature		96.0 $\pm$ 0.8	87.8 $\pm$ 6.3	89.1 $\pm$ 1.4	91.0 $\pm$ 2.3
Maturing		73.6 $\pm$ 0.0	87.8 $\pm$ 5.6	95.7 $\pm$ 4.4	85.7 $\pm$ 4.5
Population		89.3 $\pm$ 0.6	87.8 $\pm$ 3.4	91.6 $\pm$ 0.8	89.5 $\pm$ 1.2
Predicted maturation rate		24.9 $\pm$ 0.6	31.1 $\pm$ 6.1	43.7 $\pm$ 2.5	33.2 $\pm$ 3.9
Error on maturation rate		-5.1	+6.1	+5.0	+2.0 $\pm$ 3.6
• <b>Composition of the cohort predicted as maturing using discrete site datasets (%)</b>					
Maturing		88.7 $\pm$ 2.0	72.5 $\pm$ 9.8	84.7 $\pm$ 1.0	81.9 $\pm$ 4.0
Immature		11.3 $\pm$ 2.0	27.5 $\pm$ 9.8	15.3 $\pm$ 1.0	18.1 $\pm$ 4.0
• <b>Composition of the cohort predicted as immature using discrete site datasets (%)</b>					
Immature		89.5 $\pm$ 0.1	95.7 $\pm$ 1.6	97.1 $\pm$ 2.9	94.1 $\pm$ 1.7
Maturing		10.5 $\pm$ 0.1	4.3 $\pm$ 1.6	2.9 $\pm$ 2.9	5.9 $\pm$ 1.7
<b>(b.) PREDICTOR: BW</b>					
• <b>Correct prediction from own site dataset (%)</b>					
Immature		85.5	91.1	84.9	87.2 $\pm$ 2.0
Maturing		73.6	75.6	80.4	76.5 $\pm$ 2.0
Population		81.9	87.2	83.2	84.1 $\pm$ 1.6
• <b>Correct prediction from discrete site datasets (%)</b>					
Immature		92.0 $\pm$ 3.25	91.5 $\pm$ 6.3	62.4 $\pm$ 3.5	81.9 $\pm$ 6.5
Maturing		58.5 $\pm$ 11.3	66.7 $\pm$ 8.9	90.2 $\pm$ 1.1	71.8 $\pm$ 7.1
Population		82.0 $\pm$ 1.15	85.3 $\pm$ 2.5	73.1 $\pm$ 1.7	80.1 $\pm$ 2.5
Predicted maturation rate		23.2 $\pm$ 5.7	23.1 $\pm$ 6.9	58.0 $\pm$ 2.5	34.7 $\pm$ 7.7
Error on maturation rate		-6.8	-1.9	+19.3	+3.5 $\pm$ 8.0
• <b>Composition of the cohort predicted as maturing using discrete site datasets (%)</b>					
Maturing		76.6 $\pm$ 4.1	76.3 $\pm$ 13.4	60.2 $\pm$ 1.0	71.0 $\pm$ 5.0
Immature		23.4 $\pm$ 4.1	23.7 $\pm$ 13.4	39.7 $\pm$ 1.0	29.0 $\pm$ 5.0
• <b>Composition of the cohort predicted as immature using discrete site datasets (%)</b>					
Immature		84.1 $\pm$ 3.2	89.3 $\pm$ 1.9	97.1 $\pm$ 2.9	88.1 $\pm$ 1.7
Maturing		15.9 $\pm$ 3.2	10.7 $\pm$ 1.9	2.9 $\pm$ 2.9	11.9 $\pm$ 1.7



maturity cohorts characterized from a discrete site. Conversely  $28.2 \pm 9.5\%$  of truly recruited fish were below this BW threshold leading to an actual maturation rate of  $11.9 \pm 1.7\%$  in this cohort predicted as immature, compared to  $5.9 \pm 1.7\%$  using BW, FL and K for prediction and  $31.2 \pm 4.0\%$  in the initial population. The potential for cohort segregation using the optimal BW threshold characterized from another discrete population is illustrated on Fig.4.



**Figure 4.** Cumulative weight-structure diagram of the population addressed in Fig.3b. (Site A, June and July sample pooled), classified per maturity status and presented with the optimal segregating BW determined using a discrete site dataset (Site B) and BW as sole predicting factor.

In a similar fashion, we applied discriminant analysis in the female population with a GSI below 1% and harvested in June and July to assess the power of BW, FL, K in conjunction with GSI in predicting their vitellogenic status. Applying discrete site datasets, the vitellogenic status of on average  $97.7 \pm 1.2\%$  of the populations (female with  $GSI < 1\%$ ) and  $83.6 \pm 10.4\%$  of the corresponding recruited females were correctly classified using GSI as sole predictor. No significant improvement arose from adding BW, FL and K as predicting factors (Data not shown).

## 4. Discussion

### 4.1. Sexual Size Dimorphism (SSD) at the immature stage

Sexual size dimorphism (SSD) was reported in various teleosts at the post-juvenile stage but often without indications of individual maturity status (Saillant *et al.*, 2001; Young, 2005; Hanson *et al.*, 2008). Species identified as exhibiting SSD specifically at the immature stage are comparatively scarce and include sea bass (*Dicentrarchus labrax*, Saillant *et al.*, 2001), European eel (*Anguilla anguilla*, Degani *et al.*, 2003) and yellow perch (*Perca flavescens*, Shewmon *et al.*, 2007).

Atlantic salmon is usually considered monomorphic prior to sexual maturation (Kadri *et al.*, 1997a) but our data unexpectedly unveiled a significant SSD at the immature stage in this key aquacultural species. Surveyed 15 to 22 months post-sea transfer in different populations of siblings, immature males consistently achieved at each sampling point a higher body mass (+13.4%) and length but exhibited a lower K than immature females. If confirmed, SSD in immature Atlantic salmon is an important species characteristic. Occurring SSD are partly responsible for variations in individual performance in mixed-sex teleost stocks, a critical factor under experimental conditions with negative implications under commercial management (Fontaine *et al.*, 1997). The overall apparent reduced feed intake in larger individuals in three sites surveyed, as shown by the negative allometry in mean-b parameter (Arslan *et al.*, 2004), was not observed in any gender considered separately and therefore was primarily due to SSD. Importantly, the described SSD highlights that growth performances are affected by the stock sex-ratio such that genders should be addressed separately in experimental studies. Sex-ratio is an important population parameter and is likely to be skewed following weight-grading in sexually dimorphic populations. This was shown in sea bass (Papadaki *et al.*, 2005) and in this study by the power of BW to discriminate

between genders in association with the seasonality in sex-ratio observed at harvest. Significant dimorphism most probably occurs at the time of weight-grading leading to a higher proportion of males in the best performing cohorts harvested earlier in the season. If occurring at the freshwater stage, SSD could likewise lead to skewed sex-ratio in graded smolt populations with consequences on subsequent performance at sea. Finally, our data highlight a potential advantage for male-monosex stocks toward a shorter production cycle or a higher individual weight at harvest but also a reduced size variability. Nonetheless male weight advantage was on average below 15% and counterbalanced by their lower K in this study, which could result in their higher downgrading rate at processing. Furthermore, such monosex stocks would yield an increased risk of pre-harvest maturation since different studies have reported a higher rate of early maturation in males (Kråkenes *et al.*, 1991; Oppedal *et al.*, 2003).

Taken together, SSD should be further assessed in different populations of immature Atlantic salmon to determine if it is a characteristic of the species with global implications. Further investigations are also required to describe its time of onset but also its origin which could be alimentary and/or metabolic (feed level, intake and utilization) and/or if it may be linked to a sex-specific maturation strategy ahead of actual sexual recruitment as addressed in other species (Fontaine *et al.*, 1997; Imsland *et al.*, 1997).

#### **4.2. WLR and K seasonality of the immature cohort**

Regardless of existing or potential dimorphisms, gender/maturity cohorts are seldom distinguished in teleost growth studies. An isometric growth type is common in the adult stanza of teleosts fish (Wootton, 1990) and was recently reported in Atlantic cod (Árnason, 2007), brown trout (*Salmo trutta*, Arslan *et al.*, 2004), golden grey mullet

(*Liza aurata*, Ilkyaz *et al.*, 2006) and various breams (Mehanna, 2007; Chilari *et al.*, 2006; Türkmen and Akyurt, 2003). However, actual field data on Atlantic salmon WLR could not be obtained from the literature.

Sampled from four distinct sea-sites over a seven month period, WLR of the mixed-sex, single strain immature population and of each sex analyzed separately was isometric but K had a tendency to increase over the harvest season. This shows that immature individuals displayed a constant shape over the harvest period but not over time, their growth in weight being overall proportionally superior to their growth in length from spring to winter under an increasing temperature. This is consistent with previous studies in which K seasonality was linked to a lower growth in weight than in length in periods of low water temperature (Oppedal *et al.*, 1999; 2003; 2006). Photoperiod was also shown to affect growth pattern with stocks exposed to LL exhibiting a higher rate of muscle fibre recruitment and ultimately an improved K (Johnston *et al.*, 2003; Oppedal *et al.*, 2006). In our survey, immature fish harvested in June and July and showing the lowest K at harvest (Table 3b) were not exposed to LL unlike stocks harvested from August onwards. The commercial practice of applying LL only to stocks to be harvested over the reproductive season, i.e. during second half of the year when water temperature is higher, is likely to further accentuate K seasonality inherent to the seasonal pattern of weight-length development. Finally, K will also vary with the composition of the harvest batch, i.e. sex-ratio and maturation rate, due to size dimorphism between cohorts. With males exhibiting a lower K than females, mean-K of the stock would increase with the decreasing rate of males along the harvest season in weight-graded populations. In addition, the proportion of early maturing fish would positively affect mean-K of the stock in June and July when they exhibited a strong

advantage in K as reported in this study (+20.6%) and previously (Aksnes *et al.*, 1986; Kadri *et al.*, 1997a).

Overall, this analysis highlights that a population or species with an isometric WLR can also exhibit seasonal variations in K and that the apparent condition of the stock is in practice also affected by its composition (sex-ratio and maturation rate) and probably history of LL exposure, both of which vary with harvest time under typical commercial management. Such factors should also be considered before strict conclusion on feeding performances.

### **4.3. Implications of size dimorphism between maturity cohorts**

It is well recognized in Atlantic salmon that some threshold of size and/or body condition must be surpassed during the spring window for sexual maturation to proceed (Thorpe, 1986, 1989). In the current study, sexually recruited individuals had in June and July a strong advantage in BW and K but not FL over the immature cohort. Although they already initiated sexual development, our data shows that a higher growth in weight but not in length, hence a higher K, is likely critical for recruitment into maturation in spring over the second year at sea. Following recruitment, a surge in appetite and growth characterize individuals initiating sexual development during the so-called anabolic window and lead to significant discrepancies in weight-length parameters between maturity cohorts (Aksnes *et al.*, 1986; Kadri *et al.*, 1996).

While relative condition factor and FL have previously been identified as strong but population specific predictors of early sexual maturation (Kadri *et al.*, 1997a), our survey yielded BW, K and FL as standard predictors among different rearing units. Importantly, stocks from each site were from the same strain, reared together at the freshwater stage and transferred to sea within the same period. At sea, they experienced

the same overall feeding management and temperature profiles but strong variations in water clarity and salinity (strong to moderate). Such differences in salinity profiles could have led to growth discrepancies between sites due to the energetic cost of osmoregulation (Boeuf and Payan, 2001). However, temperature was shown to be the main factor influencing Atlantic salmon growth with no effect of strong to moderate salinity (Duston, 1994; Usher *et al.*, 1991; Handeland *et al.*, 1998). Furthermore, different growth patterns are observed in smolts transferred to sea at different times of the year (Duncan *et al.*, 1998). In our survey, the proximity of the life-cycle history and the common genetic origin was undoubtedly critical in the occurrence of standard morphological attributes among our discrete sites which constituted in that sense a cluster of rearing units.

These results highlight the possibility of implementing a practical maturation management strategy during the anabolic window of the reproduction cycle. Within identified rearing clusters, characterization of size dimorphism between maturity cohorts can be restricted to a “model” population then directly extended to discrete units to estimate their maturation rate on-site by simple weight-length assessment of the stock, an approach that correctly identified over 85% of recruited individuals in this study. It must be acknowledged that using the body size dimorphism characterized in the specific rearing unit increases the accuracy of the predicted maturation rate. This might prove worthwhile particularly with the possibility to estimate maturity status of sacrificed females based on their GSI only, as shown by the common GSI threshold between non-vitellogenic and vitellogenic ovaries in our discrete populations. Following maturation rate estimation, simple weight-grading has the potential if required to mechanically segregate a high proportion of maturing fish. As shown in the case illustrated in Fig.4, top-grade harvest based on the optimal cut-off weight

determined in a discrete site would have segregated 80% of the recruited individuals in 38% of the whole stock. However, nearly 90% of maturing fish were among the heaviest half of the stock highlighting that, in practice, lowering the statistically optimal cut-off weight could increase the selective harvest of maturing fish.

Thanks to its restricted reliance on invasive sampling, this maturation detection-segregation strategy can be readily implemented by the Atlantic salmon industry with twofold advantages. Primarily an increased output of quality biomass by harvesting early maturities when yielding superior quality characteristics but also an improved growth, feeding and welfare of the immature stock left for further on-growing. However, the feasibility and economic interest of selectively harvesting maturities will likely be significant over some threshold of maturation rate hence the importance of its prior estimation. The widespread implementation of this detection-segregation strategy for managing sexual maturation at sea requires confirmation that significant body size dimorphisms occur in different strains farmed under a variety of conditions along with a better knowledge of the genetic (e.g. strain, families, generations), environmental and husbandry parameters resulting in standard size dimorphism between discrete populations. A more thorough set of morphometric indicators could also increase the accuracy of maturation rate estimation since body depth at the point where the anal fin arises was shown to be a significant predictor of early maturation in Scottish Atlantic salmon (Kadri *et al.*, 1997a). Importantly, the window of opportunity for implementation of this management strategy was identified as June and July and closed from August onward but could occur earlier in the spring. Finally, biomass scanning technologies (e.g. Vaki, Storvik) could greatly facilitate the monitoring of maturation onset in salmon stocks by assessing a high number of morphological predictors in a more representative sampling size *in situ*. Likewise, stock grading based on a number of

external morphological parameters could improve the selective harvest of the maturing cohort in comparison to simple weight-grading but no equipment is presently available to do so.

The negative impact of sexual maturation on product marketability and growth is common among most commercially important species (Kjesbu *et al.*, 1991; Bromage *et al.*, 2001; Felip *et al.*, 2001; Almansa *et al.*, 2001; Grigorakis, 2007; Roth *et al.*, 2007) while age at first maturity varies according to sex in various teleosts such as sea bass (Felip *et al.*, 2008). In this latter species, repetitive weight-grading of mixed-sex populations allowed segregating a fast-growing, 96.5% female, population for experimental purpose (Papadaki *et al.*, 2005). Mechanical segregation of genders and sex-specific harvest could minimize maturation during sea bass on-growing to optimize biomass output and facilitate the production of various market sizes. Similarly in turbot (*Scophthalmus maximus*), it was recommended to segregate non-maturing fish by size grading but also to develop methods for the production of all-female stocks to optimize growth performance (Imsland *et al.*, 1997). Dimorphism-based management strategies are likely to prove beneficial in various aquacultural species and modeling of weight-length dimorphism among rearing units to facilitate their commercial implementations.

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## CHAPTER 4

### PAPER IV

#### **RESEARCH ARTICLE**

#### **SEASONAL VARIATIONS IN SKIN PIGMENTATION AND FLESH QUALITY OF ATLANTIC SALMON (*SALMO SALAR*, L.): IMPLICATIONS FOR QUALITY MANAGEMENT.**

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**Keywords:** Atlantic salmon, skin pigmentation, coloration, flesh quality, sexual maturation, reconditioning.

**Note:** Letters of references “Leclercq et al. 2010a, d” are not alphabetically in the thesis format of this manuscript (MS) due to cross-references in other published MS within the present thesis.

## **Abstract**

The external coloration of fish is a key driver in the consumer buying decision and is typically altered during sexual maturation in salmonids. Farmed Atlantic salmon (*Salmo salar* L.) exhibiting distinct phenotypes from the typical silver and nuptial colouration were described in terms of sexual development, flesh quality and skin pigment profiles. Reconditioning of skin colouration during storage was also tested (CIE[1976]L\*a\*b\*) with the overall view of optimizing quality management. The intermediary phenotype never reflected significant deteriorations in flesh quality. It originated from a lack of purine pigments (guanine and hypoxanthine) revealing the carotenoid compounds dominated by the yellow-orange  $\beta$ -carotene. The resulting distinctive lightness and yellowness were reduced by direct ice contact at a post-mortem stage. Storage conditions can be optimized to improve and standardize the coloration of whole-fish yielding superior flesh quality parameters. This would facilitate product quality grading during primary processing but also increase product acceptance and attractiveness.

## **1. Introduction**

The external coloration of fish is an instinctive indicator of product freshness and quality hence constitutes a key driver in the consumer buying decision (Robb, 2001). This is true for both skin and flesh color in many aquaculture species that are sold whole or processed. From Atlantic salmon (*Salmo salar* L.), the consumer expects a deep pink-red flesh and a silvery-blue skin characteristic of the sea-running immature life-stage. Fish skin coloration is a complex trait generated by four main types of pigment stored in interacting chromatophores. Melanin, carotenoids and pteridines are true light-absorbing pigments found primarily in black-brown melanophore, red-orange erythrophore and ochre-yellow xanthophore respectively. In contrast, iridophore and

leucophore are light-reflecting chromatophores generating a blue-green-iridescent and white-creamy colors respectively due to variations in the structural organization of their purine based crystalline organelles (Fujii; 2000). Alterations in the distribution, morphology or density of chromatophores and/or in the concentration of their pigment granules define morphological skin color changes. They are slow and long-lasting phenomena elicited in response to environmental (e.g. surrounding light-conditions) and social (e.g. subordination; territoriality) factors but also observed during life-stage transitions such as smoltification and nuptial metamorphosis in salmonids (Leclercq *et al.*, 2010d). In contrast, physiological skin color changes occur from the migration i.e. dispersion or aggregation of pigment-vesicles within their cells. They are acute and transient events stimulated by neuronal and endocrine factors (Fujii; 2000) and can occur at a post-mortem stage (Doolan *et al.*, 2008; Erikson and Misimi, 2008). The coloration of salmonid muscle originates from carotenoid pigments fed in the form of astaxanthin (Ax) and canthaxanthin (Cx) through well-defined pigmentation feeding regimes during on-growing (Bjerkeng *et al.*, 1992). Although a variety of nutritional factors affect flesh carotenoid deposition and visualization (Nickell and Springate, 2001), sexual maturation has the most extensive effect under standard farming practices. Carotenoid pigments are in fact accumulated in the flesh at the immature stage and redistributed to both the skin and gonads over the course of sexual maturation (Bjerkeng *et al.*, 1992; Nickell and Springate, 2001; Torrissen and Torrissen, 1985; Rajasingh *et al.*, 2006). The development of nuptial coloration is therefore used as an indicator of reduced flesh carotenoid but also lipid and protein levels, also due to the reproductive effort (Aksnes *et al.*, 1986), during product quality grading.

Holding both ecological and economic interests, nuptial metamorphosis is the best studied phenomenon of skin color change in salmonids but only with regards to

carotenoid compounds. Melanin pigments could confer nuptial salmonids their typical dark-grey appearance while purine compounds are likely to be altered during desmoltification associated with sexual maturation. Skin silvering during smoltification positively correlated with purine concentration and alteration in osmoregulatory parameters (Johnston and Eales, 1967; Haner *et al.*, 1995). The focus given to carotenoid pigments is likely to restrict understanding of color expression in Atlantic salmon. With virtually no other factors described as recurrently affecting the skin coloration of farmed Atlantic salmon, sexual maturation and altered flesh quality are typically presumed when deviations from the desirable silver phenotype occur. This result in product downgrading toward a lower value market channel at primary processing and reduces the economic sustainability of the industry.

Sea-reared Atlantic salmon exhibiting an intermediary skin coloration between the typical silver and nuptial phenotypes, of immature and mature salmon respectively, are repeatedly observed at harvest. These fish display a pearly-white belly characteristic of immature fish but a pale-greenish back resembling the instigation of the nuptial metamorphosis. This phenotype could be expected in spring/early summer during the recognized window for initiation of sexual maturation but is observed over the whole reproductive season. The present study was therefore undertaken to confirm, quantify and characterize such altered phenotypes in terms of sexual development, flesh quality and skin pigment profile in comparison to the typical silver-immature and nuptial-mature skin coloration. Feasibility of their post-harvest reconditioning was also assessed. The aim of this study was to improve understanding of color expression in Atlantic salmon in relation to flesh quality with the view to optimize post-harvest quality management.

## 2. Materials and methods

### 2.1. Animals and sampling

Atlantic salmon were reared under standard commercial management up to harvest size in one Scottish sea-site (Lat: 56°39'19'', Long: -5°19'56''). The stock was fed a commercial diet according to manufacturer recommendations (35% protein, 32% lipids, 16.5% carbohydrates, 10 ppm Ax and 5 ppm Cx; Skretting, Invergordon, UK). Sampling was performed once a month from June to December 2007 at the processing plant, 8 h to 12 h post-mortem. Up to three distinctive phenotypes were subjectively observed: silver, intermediary (white belly and pale-green back) and nuptial. Their prevalence was determined by a single observer with a minimum of 600 observations from at least three counting sessions over the processing period. Within each skin color category, 30 fish/gender/skin color category were randomly collected and measured for whole body weight (BW) ( $\pm 0.1$  g), fork length (FL) ( $\pm 0.1$  cm) and gonad weight (GW) ( $\pm 0.01$  g). Fulton condition factor (K) and gonado-somatic-index (GSI) were calculated as follows:  $K = (100 \text{ BW}) \text{ FL}^{-3}$  and  $\text{GSI} (\%) = (100 \text{ GW}) \text{ BW}^{-1}$ . Female ovaries were preserved in Bouin's fixative for 24 h then in 70% ethanol before processing for histological observation. Among these fish, the left-hand side flesh Scottish quality cut (SQC), representing the steak taken immediately below the dorsal fin, were excised from 10 female/skin color category and stored at - 20 °C until quality analysis. In December, a further 10 fish/skin color (1:1 sex-ratio) were collected for flesh, skin and gonad pigment analysis following assessment of morphological and gonadal parameters. The left-hand side SQC was carefully skinned using a scalpel. Skin samples from a standardized area of the dorsal and ventral SQC were excised and divided vertically in two for carotenoids and melanin, and purine content analysis respectively. The skinned SQC, skin and gonad samples were frozen at - 20 °C until carotenoid

analysis. Finally, post-harvest skin color reconditioning was assessed using 5 silver and 5 intermediary colored fish randomly sampled in December. Each fillet from each fish was cut into 3 transverse sections (flesh quality cut, SQC, Norwegian quality cut; Johnston *et al.*, 2007) then rinsed in distilled water before testing for reconditioning.

## 2.2. Sexual development and flesh quality analysis

Males were classified as immature or sexually recruited based on their bimodal GSI frequency distribution in the population with a threshold value of  $GSI = 0.20\%$  (Kadri *et al.*, 1997a). Female ovary samples were classified according to their leading oocyte stage with onset of true exogenous vitellogenesis used as indicator of recruitment into sexual maturation (Taranger *et al.*, 1999). Flesh SQCs were thawed overnight, skinned and deboned prior to analysis. Flesh color was scored by two independent assessors under standard light condition using the Roche *SalmoFan*<sup>TM</sup> lineal color card for salmonids, scale 20-34 (Hoffman-LaRoche Ltd.; Basel, Switzerland) in the dorsal, midline and belly regions then averaged. Flesh color composition (CIE[1976]L\*a\*b\*) was measured in the same areas using a tristimulus colorimeter (Minolta Chroma Meter, CR-310, Minolta Corporation; Osaka, Japan) against a standard calibration tile (Robb, 2001). Flesh samples were then homogenized for analysis of total carotenoids, total lipid and lipid proximate composition by near infrared reflectance spectroscopy (NIR) (FOSS 6500 NIR analyser, Foss NIR Systems, Foss UK Ltd.; Didcot, UK) (Fjellanger *et al.*, 2001). The NIR analyser was calibrated daily prior to analysis using a calibration check cell and twice over the analysis period against wet chemistry (HPLC).

**Pigment extraction**

For carotenoid analysis (December samples), skin samples were weighted ( $\pm$  0.001 g) and their surface area determined by image analyses (Image ProPlus 4.5, Mediacybernetics, MD) using a stereomicroscope then homogenized for carotenoid extraction. Skinned SQCs and gonads were thawed overnight, homogenized (immature male gonads were pooled per skin color category due to low tissue weight and expected carotenoid concentration) and 1 g of tissue separated for extraction of carotenoids as follows (Barua *et al.*, 1993; Torstensen *et al.*, 2005). Tissue samples were placed in a stoppered glass tube containing 10 ml of absolute ethanol:ethyl acetate mixture (1:1; v/v) and homogenized using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). The homogenate was centrifuged (1000 g, 5 min) and the supernatant removed to a clean stoppered glass tube. The pellet was re-homogenized in 5 mL of ethyl acetate then in 10 mL of isohexane and the supernatants pooled following centrifugation (1000 g, 5 min). The pooled supernatant was evaporated to dryness under a nitrogen (N<sub>2</sub>) flow and vacuum desiccated in total darkness overnight. The residue was finally redissolved in 5 ml to 20 ml isohexane containing 0.2% (w/v) butylated hydroxytoluene (BHT) before injection into the HPLC system. Skin melanin was extracted from the carotenoid stripped skin pellet which was immediately resuspended in 10 ml 0.2% NaOH and stored overnight at - 4 °C. The sample was then boiled in 0.2% NaOH for 1h with regular mixing. The solution was centrifuged (2100 g; 5 min), the supernatant removed to a stopper glass tube and the skin pellet re-extracted as previously described. Supernatants were combined, topped-up to 20 ml precisely with 0.2% NaOH and mixed. A 2 ml fraction was centrifuged (6100 g; 5 min) and measured by spectrophotometry at 340 nm against a synthetic melanin standard (Sigma-Aldrich Ltd., Poole, UK) pre-solubilized in 1 ml 1 M NaOH and 10  $\mu$ L 3% H<sub>2</sub>O<sub>2</sub> by heating in a

boiling water bath for 30 min (Szisch *et al.*, 2002). Purine extraction protocol was modified from Oliphant (1987a) as follows. Surface area and weight of the skin samples were determined as previously described then homogenized with an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK) in 10 ml 0.1 M NaOH. The stoppered glass tube was placed in an ultrasonic bath for 20 min, regularly mixed for another 40 min and the supernatant separated by centrifugation (220 g, 5 min). The skin pellet was re-extracted twice in 5 ml 0.1 M NaOH and a final time in 10 ml 0.1 M NaOH following overnight storage at - 4 °C. The final supernatant was translucent and supernatants combined for a total extract volume of 30 ml. A 2 ml fraction was diluted in 2 mL distilled H<sub>2</sub>O providing a total dilution factor of 60 and a 0.05 M NaOH extract. This aliquot was mixed and centrifuged (6100 g; 5 min) before injection into the HPLC system.

### **HPLC systems**

The chromatographic system used for carotenoid analysis was previously described (Torstensen *et al.*, 2005). It consisted of a 5 µm ODS2 column (4.6 x 150 mm, Phenomenex, Macclesfield, U.K.) equipped with a Waters model 501 pump and a Waters 490E multiwavelength UV-vis detector (Millipore, Watford, U.K.) for detection at 450 nm. An isocratic solvent system containing ethyl acetate/methanol/water (20:72:8 v/v/v) at a flow rate of 1 ml min<sup>-1</sup> was used and carotenoid compounds were quantified using as external standards Ax, Cx and β-Carotene (DSM Nutritional Products, Basle, Switzerland). Purine (guanine; hypoxanthine; adenine; Sigma-Aldrich Ltd., Poole, UK) and pteridine standards (xanthopterin, sepiapterine, leucopterin, xanthine, biopterin, isoxanthopterin, lumazine; Schircks Laboratories, Jona, Switzerland) were dissolved in 0.05 M NaOH at concentrations ranging from 0.2 to 1 mg ml<sup>-1</sup> according to manufacturer recommendations. Serial dilutions to concentrations of 1 µg ml<sup>-1</sup>, 2 µg ml<sup>-1</sup>



<sup>1</sup> or 5  $\mu\text{g ml}^{-1}$  were performed prior to injection into the HPLC system. For analysis of purine compounds, the chromatographic system consisted of a constaMetric 4100 pump equipped with a UV 3000 detector and peak areas were calculated using ChromQuest software (ThermoFisher, Hemel Hempstead, UK). The mobile phase consisted of a mixture of 0.01 mM potassium phosphate buffer, pH 3.2 using phosphoric acid, with 4% methanol. Pigments were eluted isocratically at a flow rate of 1 ml  $\text{mn}^{-1}$  and detected at 254 nm after elution from a Partisil-10 ODS-1 column (250 x 4.6 mm; Phenomenex, Macclesfield, UK; modified from Hudon and Muir, 1996; Porcar *et al.*, 1996). This method for purine analysis was expected to be equally effective for pteridine analyses, as described by these authors in other tissues. Pteridine standards were revealed but no pteridine compounds were identified in our samples. This was possibly due to the low concentration of pteridine compounds in teleost integuments along with the high sample dilution factor required for analysis of purines found at high concentration in teleost skin.

### **Skin color reconditioning**

Five cuts from the same fish were randomly placed skin down in direct contact with ice of different composition (Freshwater (FW); FW +0.25 M  $\text{Na}^+$ ; FW +0.5 M  $\text{Na}^+$ ; FW +0.75 M  $\text{Na}^+$ ; FW +5 mM caffeine) and the last cut was placed in direct contact with skin by juxtaposing another sample. Caffeine is known to induce melanophore dispersion at this concentration (Rodionov *et al.*, 1998). These treatments are later referred to as FW, Na1, Na2, Na3, CAF and Skin contact respectively. Each treatment comprised five samples from five different fish/color category except for skin contact treatment which received four samples. The different ice baths were placed in a styrofoam box to maintain a constant ice temperature throughout the experiment. Skin color composition was measured immediately before treatment application (T0) then at

T0 +6 h (T1), T0 +24 h (T2) and T0 +30 h (T3) by means of tristimulus colorimeter as described previously for flesh color analysis. Skin color was assessed in triplicate in both the dorsal and belly region then averaged per body area. With  $a^*$ -value (red-greenness) and  $b^*$ -value (yellow-blueness) close to achromatic in the dorsal skin, minor changes in those parameters led to high and irrelevant changes in hue ( $H_{ab}$ ) and chroma ( $C_{ab}$ ) (Doolan *et al.*, 2008) such that focus was given to primary  $L^*a^*b^*$  parameters of color composition.

### 2.3. Statistics

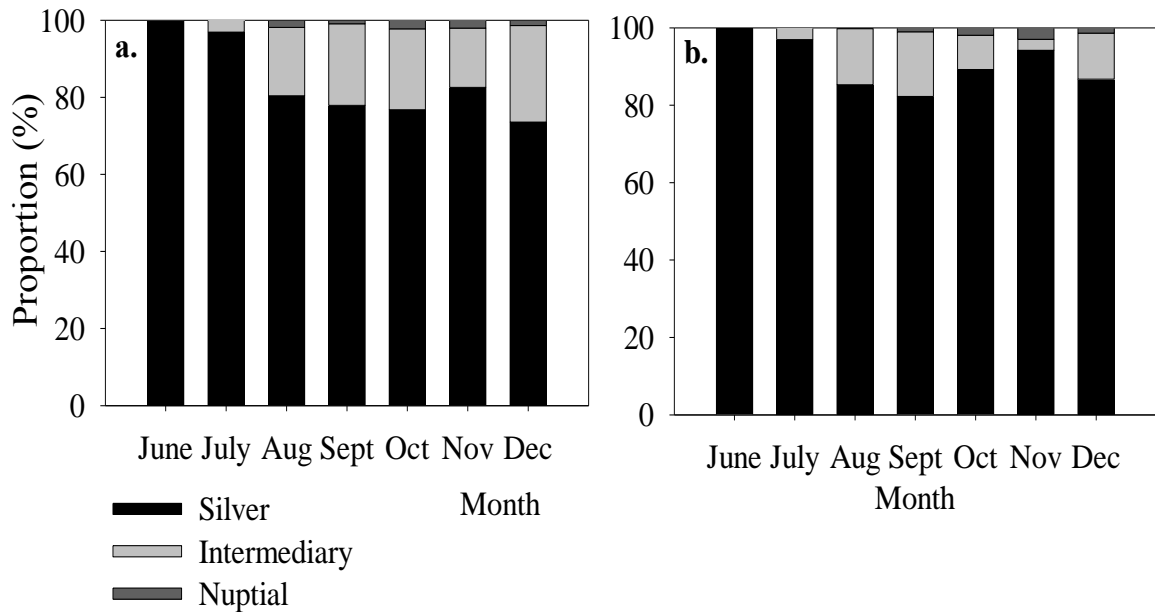
Prior to statistical analysis using Minitab v.15, datasets were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test, examination of residual plots), log-transformed and proportions arcsin transformed when required. Differences in morphological, GSI and flesh quality parameters between color groups were tested at each sampling point by one-way analysis of variance (ANOVA). For December samples for pigment analysis, parameters were analyzed by three-way ANOVA to test the effect of skin color, body area and sex on skin pigment concentration and compound proportions and by two-way ANOVA to test the effect of skin color and sex on morphological parameters, skin dorso-ventral pigment ratio, flesh and gonad carotenoid content. Parameters of skin color composition assessed in the post-mortem reconditioning challenge were analyzed using a three-way repeated measure ANOVA manipulated by a General Linear Model (GLM) to test the effect of treatment, storage time and skin color category in each body area. In each color group, parameters were never statistically different at T0 i.e. between on-coming treatments. Samples were pooled at this time point to characterize, for each body area, differences in color composition between color groups (one-way ANOVA). Furthermore for each

color group, there were no statistical differences between treatments in the ventral area or between the five ice quality tested in the dorsal area (FW, Na1, Na2, Na3, CAF). Data were pooled accordingly to compare, in particular, the effect of ice and skin contact in the dorsal area. Where statistical differences were found ( $P < 0.05$ ), post hoc multiple comparisons were applied using Tukey's test. All data are expressed as mean values  $\pm$  standard error of mean (SE).

### **3. Results**

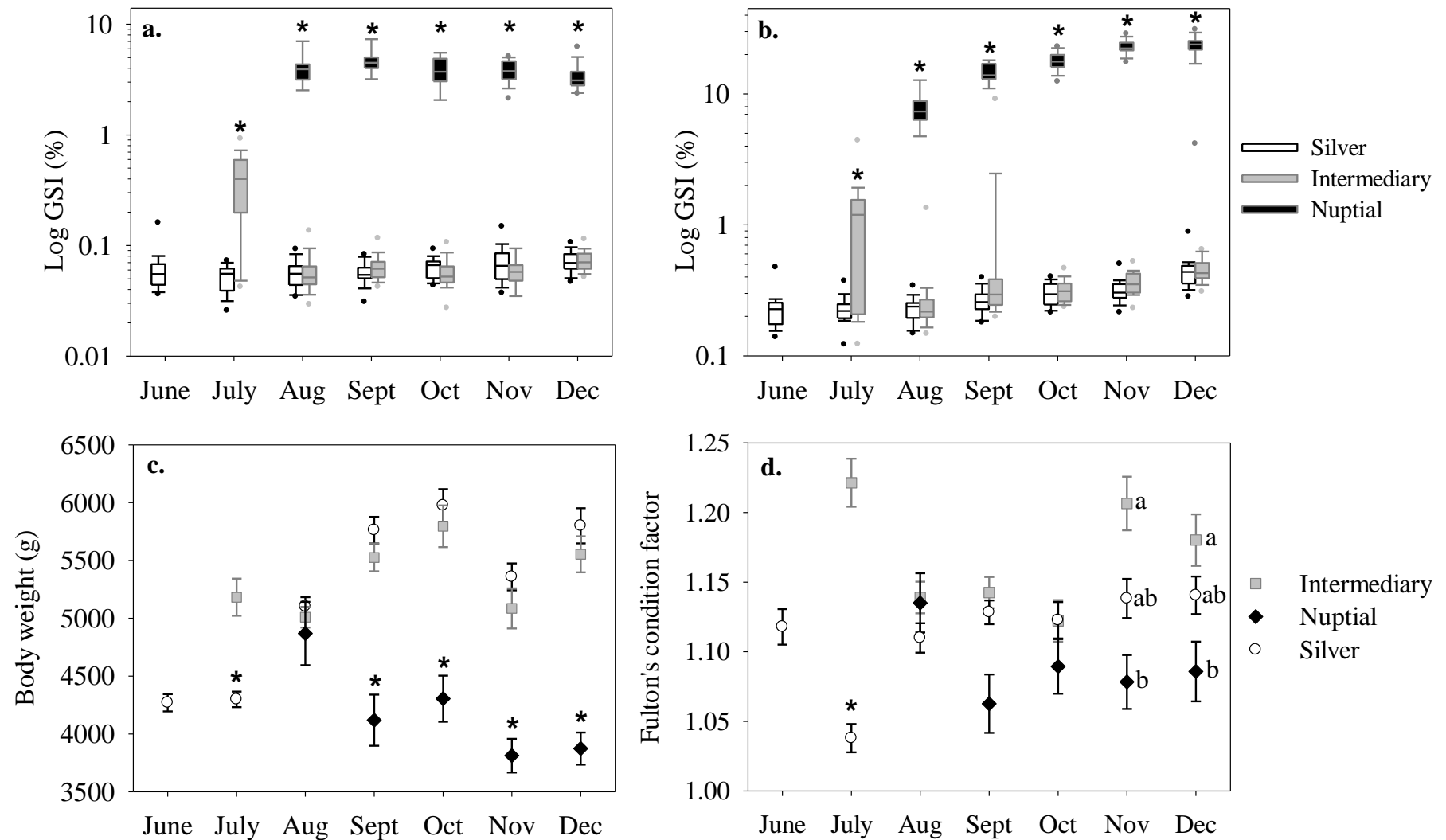
#### **3.1. Seasonal occurrence, sexual development, and flesh quality of observed phenotypes.**

The proportion of the different color groups observed at harvest is presented in Fig.1. Intermediary fish were observed from July until December. Their incidence increased over the summer accounting for 3% in both the male and female cohorts in July but for 17% and 21% in those respective genders in September. Comparatively less frequent in October and November, 12% and 25% of harvested males and females exhibited a distinctive intermediary coloration in December. Nuptial fish were observed from August, a month later than the first occurring intermediary fish, to December with a monthly prevalence rate always below 3%.



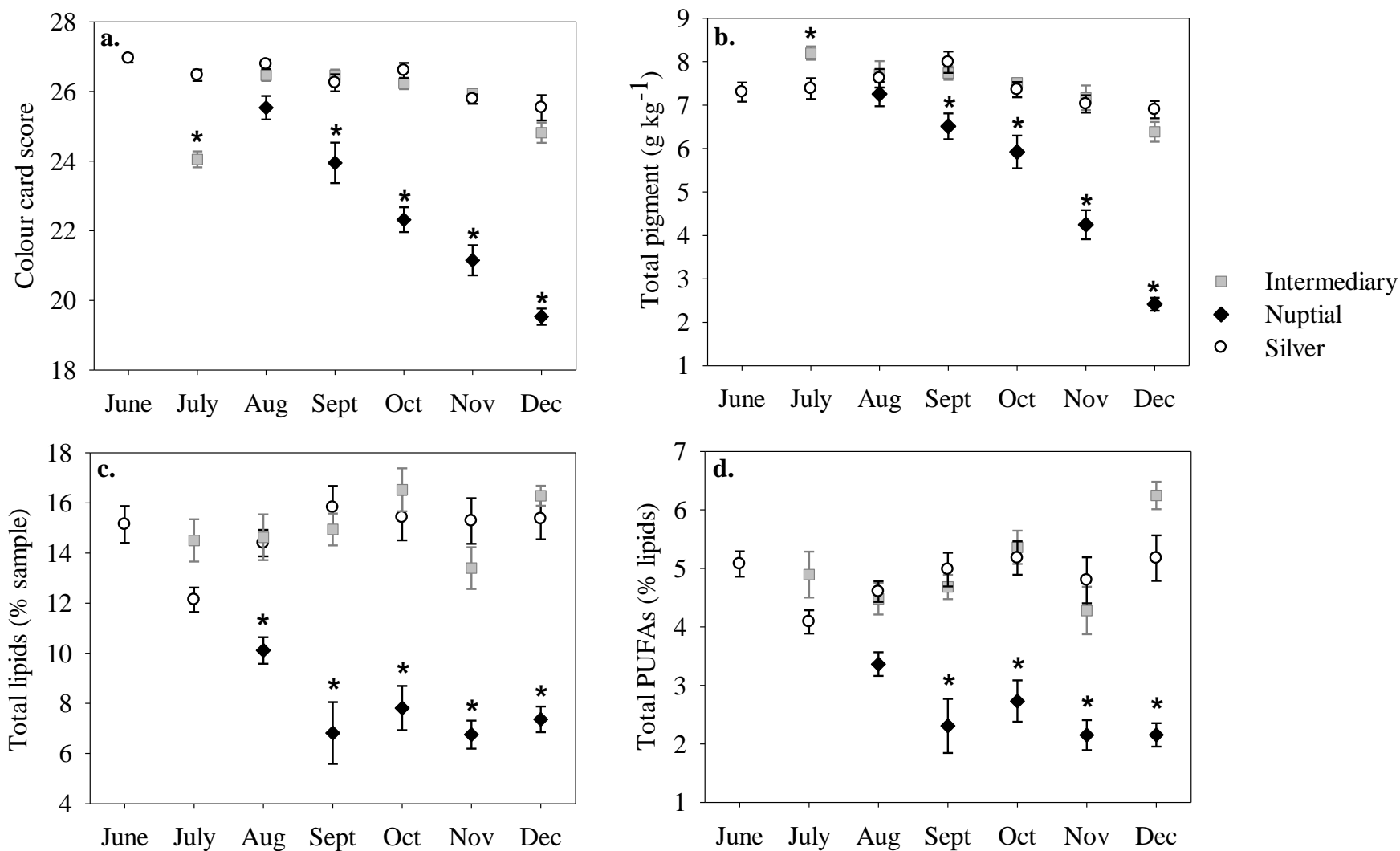
**Figure 1.** Proportion of the three skin colour categories observed at harvest in (a.) female cohort and (b.) male cohort (n = 30 fish/sex/colour group/sampling point).

Histological evidence of recruitment into sexual maturation was first observed in July in fish exhibiting an intermediary skin coloration (Male-GSI: 0.2% to 0.9%, female-GSI: 0.72% to 2.0% at the primary and secondary yolk stage). Intermediary males and females had a significantly higher GSI than their silver siblings in July (Fig.2a, 2b), although not all but 77% and 63% of the intermediary males and females were sexually recruited (data not shown). Silver and intermediary males were systematically immature from August to December as shown by a maximum GSI of 0.16% (Fig.2a). Similarly, there were no significant differences in GSI between silver and intermediary females over this period (Fig.2b). However, while silver females were always immature from August to October, 6.7% and 17% of the intermediary females were at an exogenous vitellogenic stage in August and September respectively (data not shown). Later in November and December, females at an early maturational stage (primary yolk stage, GSI < 0.72%) occurred in both the silver and intermediary cohorts and at a rate of 23% and 33% respectively in December (data not shown).



**Figure 2.** GSI box-plot distribution of (a.) male cohort and (b.) female cohort within each skin colour group (n = 30 fish/sex/colour group/sampling point). (c.) Mean body-weight and (d.) mean condition factor of each skin colour cohort. Asterisks (\*) or different superscript letters indicate significant differences between skin colour groups within each month (ANOVA, P < 0.05).

In comparison, GSIs of nuptial males and females were always significantly higher than both their silver and intermediary siblings. Over this period, GSI of nuptial males ranged from 2.3% to 7.3% and GSI of nuptial females, always at the tertiary yolk stage, between 4.7% and 31.4% (Fig.2a, 2b). Differences in morphological parameters between the silver and intermediary fish occurred in July only when the former cohort was significantly heavier (+884 g, +21%) with a higher K (+0.18, +18%) (Fig.2c, 2d). In comparison, nuptial fish were significantly lighter from September onward and their K lower than that of intermediary fish in November and December. Such differences in body morphology were concomitant with differences in flesh quality parameters assessed in females only. In July, intermediary compared to silver females had a higher total flesh lipid (+2.4g 100g<sup>-1</sup>, +20%) and carotenoid content (+0.8 mg kg<sup>-1</sup>, +11%) but their flesh color score was significantly reduced (-2.4 points, -9%; Fig.3). There were no further differences in flesh quality between cohorts over the remainder of the harvest season. In contrast, nuptial females always had significantly altered flesh quality parameters. In August, they had a significantly lower total flesh lipid content (10.1 ± 0.5%) compared to the silver (14.4 ± 0.5%) and intermediary cohorts (14.6 ± 0.9%). This was followed from September onwards by a significant reduction in all flesh quality parameters assessed (Fig.3).



**Figure 3.** Flesh quality parameters within each skin colour group. (a.) Roche colour card rating score, (b.) total pigment ( $\text{mg kg}^{-1}$ ), (c.) total lipids (% sample) and (d.) total PUFAs (% lipids). Values are expressed as mean  $\pm$  SE ( $n = 10$  female/skin colour group/sampling point). Asterisks (\*) indicate significant differences between skin colour groups within each month (ANOVA,  $P < 0.05$ ).

### 3.2. Pigment concentration and profiles

Morphological and gonadal parameters of fish sampled in December for pigment analyses are presented in Table 1. No differences in BW, K and GSI between silver and intermediary fish were observed at this time as observed in the seasonal sampling. Melanin pigments were more concentrated in the dorsal than ventral regions in all experimental groups (Table 2). This was particularly marked in intermediary females where the melanin dorso-ventral ratio was significantly higher reaching  $3.1 \pm 0.5$  compared to an average of  $1.7 \pm 0.1$  in the other cohorts. Melanin concentrations were significantly lower in intermediary females than in silver males, silver females and nuptial males in the ventral area and than in nuptial males dorsally. The highest skin melanin concentrations were measured in nuptial males in both body areas. Purine pigments were always more concentrated ventrally than dorsally with a steady dorso-ventral ratio among experimental cohorts averaging  $0.3 \pm 0.0$  (Table 2). Nuptial fish had significantly less purines in their integument than silver fish. Differences were significant for both genders in the ventral area (Male:  $-699 \mu\text{g cm}^{-2}$ , -50%; Female:  $-773 \mu\text{g cm}^{-2}$ , -58%) and for males only in the dorsal area (Male:  $-152 \mu\text{g cm}^{-2}$ , -49%; Female:  $-118 \mu\text{g cm}^{-2}$ , -39%). In comparison, intermediary fish had an intermediate purine concentration which was never different to any other cohort despite being reduced by 24.9% ( $-76 \mu\text{g cm}^{-2}$ ) dorsally and by 37.8% ( $-518 \mu\text{g cm}^{-2}$ ) ventrally when compared to their silver siblings (both gender averaged). Two purine compounds were identified, guanine (G) and hypoxanthine (H), with a strong positive linear correlation between their concentration expressed per skin surface area ( $G = 4.74 H - 142.13$ ,  $r^2 = 0.98$ ) (Data not shown). Purine profiles were never different between cohorts in the ventral area where the G/H ratio averaged  $4.1 \pm 0.1$ . The proportion of G, hence the G/H



**Table 1.** Morphological parameters of individuals sampled in December for pigment analysis. Values are expressed as mean  $\pm$  SE (n = 5 fish/sex/colour group). Different letters indicate significant differences between experimental groups (ANOVA, P < 0.05).

	Female			Male		
	Silver	Intermediary	Nuptial	Silver	Intermediary	Nuptial
Body Weight (g)	4425 $\pm$ 167 ab	4483 $\pm$ 208 ab	3803 $\pm$ 92 a	4750 $\pm$ 181 ab	5021 $\pm$ 255b	4182 $\pm$ 245 ab
Condition Factor	0.96 $\pm$ 0.07 a	1.08 $\pm$ 0.02 ab	1.16 $\pm$ 0.02 b	1.00 $\pm$ 0.03 ab	1.01 $\pm$ 0.01 ab	0.94 $\pm$ 0.03 a
GSI (%)	0.30 $\pm$ 0.04 a	0.39 $\pm$ 0.07 a	24.01 $\pm$ 2.00 b	0.07 $\pm$ 0.01 y	0.07 $\pm$ 0.01 y	3.42 $\pm$ 0.23 z

**Table 2.** Skin melanin and purine pigment concentrations and profiles. Values are expressed as mean  $\pm$  SE (n = 5 fish/sex/colour group). Different letters indicate significant differences between experimental groups. Italics denote significant differences between body areas within each experimental group for each parameter (ANOVA, P < 0.05). D, dorsal skin; V, ventral skin; G, guanine; H, hypoxanthine.

	Female			Male		
	Silver	Intermediary	Nuptial	Silver	Intermediary	Nuptial
<b>• Total melanin concentration (<math>\mu\text{g cm}^{-2}</math>)</b>						
Dorsal	288.0 $\pm$ 20.4 ab	219.7 $\pm$ 28.5 a	272.6 $\pm$ 16.4 ab	286.9 $\pm$ 22.2 ab	269.6 $\pm$ 3.1 ab	311.5 $\pm$ 9.6 b
Ventral	<i>179.2 <math>\pm</math> 17.8 a</i>	<i>80.3 <math>\pm</math> 18.8 b</i>	<i>154.4 <math>\pm</math> 13.1 ab</i>	<i>172.6 <math>\pm</math> 8.7 a</i>	<i>149.0 <math>\pm</math> 3.4 ab</i>	<i>213.8 <math>\pm</math> 16.1 a</i>
D/V	1.7 $\pm$ 0.2 a	3.1 $\pm$ 0.5 b	1.8 $\pm$ 0.1 ab	1.7 $\pm$ 0.2 a	1.8 $\pm$ 0.0 ab	1.5 $\pm$ 0.1 a
<b>• Total purine concentration (<math>\mu\text{g cm}^{-2}</math>)</b>						
Dorsal	298.9 $\pm$ 37.1 a	218.6 $\pm$ 15.7 ab	181.3 $\pm$ 28.0 ab	307.8 $\pm$ 18.6 a	237.2 $\pm$ 25.7 ab	156.2 $\pm$ 14.7 b
Ventral	<i>1338.1 <math>\pm</math> 84.0 a</i>	<i>844.5 <math>\pm</math> 114.6 ab</i>	<i>565.1 <math>\pm</math> 61.2 b</i>	<i>1398.5 <math>\pm</math> 96.6 a</i>	<i>857.4 <math>\pm</math> 175.6 ab</i>	<i>700.1 <math>\pm</math> 44.4 b</i>
D/V	0.2 $\pm$ 0.0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	0.4 $\pm$ 0.1	0.2 $\pm$ 0.0
<b>• Purine profile (%)</b>						
Dorsal						
Guanine	73.4 $\pm$ 0.8 a	69.5 $\pm$ 1.6 ab	67.3 $\pm$ 1.4 bc	73.3 $\pm$ 1.4 a	70.6 $\pm$ 1.3 ab	61.4 $\pm$ 2.4 bc
Hypoxanthine	26.6 $\pm$ 0.8 a	30.5 $\pm$ 1.6 ab	32.8 $\pm$ 1.4 b	26.7 $\pm$ 1.4 a	29.5 $\pm$ 1.3 ab	38.6 $\pm$ 2.4 c
G/H	2.8 $\pm$ 0.1 a	2.3 $\pm$ 0.2 ab	2.1 $\pm$ 0.1 bc	2.8 $\pm$ 0.2 a	2.4 $\pm$ 0.2 ab	1.6 $\pm$ 0.2 c
Ventral						
Guanine	<i>80.5 <math>\pm</math> 0.6</i>	<i>79.9 <math>\pm</math> 0.7</i>	<i>80.5 <math>\pm</math> 0.3</i>	<i>80.9 <math>\pm</math> 0.2</i>	<i>80.5 <math>\pm</math> 0.3</i>	<i>80.8 <math>\pm</math> 0.7</i>
Hypoxanthine	<i>19.5 <math>\pm</math> 0.6</i>	<i>20.1 <math>\pm</math> 0.7</i>	<i>20.3 <math>\pm</math> 0.6</i>	<i>19.1 <math>\pm</math> 0.2</i>	<i>19.5 <math>\pm</math> 0.3</i>	<i>19.2 <math>\pm</math> 0.7</i>
G/H	<i>4.1 <math>\pm</math> 0.2</i>	<i>4.0 <math>\pm</math> 0.2</i>	<i>4.0 <math>\pm</math> 0.1</i>	<i>4.2 <math>\pm</math> 0.1</i>	<i>4.1 <math>\pm</math> 0.1</i>	<i>4.0 <math>\pm</math> 0.1</i>

ratio, was significantly lower dorsally than ventrally in all experimental groups and in the dorsal skin of nuptial compared to silver fish (Male: -12%; Female: -6%; Table 2).

The last class of pigment analyzed, carotenoid compounds, was always at a higher concentration dorsally with a steady dorso-ventral ratio averaging  $1.9 \pm 0.1$  among cohorts. Carotenoids were found at similar levels in silver and intermediary fish of both genders. In males, carotenoids were significantly more concentrated in nuptial fish both dorsally ( $+2.6 \mu\text{g cm}^{-2}$ , +85.2%) and ventrally ( $+1.6 \mu\text{g cm}^{-2}$ , + 89.6%). A similar trend was observed in females but their higher carotenoid content at the nuptial stage was not significant dorsally ( $+1.4 \mu\text{g cm}^{-2}$ , +43.0%) nor ventrally ( $+0.4 \mu\text{g cm}^{-2}$ , +19.2%). At this life-stage, males accumulated significantly more carotenoids than females ventrally ( $+1.1 \mu\text{g cm}^{-2}$ , +53.3%) but not dorsally ( $+1.0 \mu\text{g cm}^{-2}$ , +20.6%). The prime carotenoid compound in the integument was  $\beta$ -carotene ( $69.9 \pm 1.4\%$ ) followed by a number of uncharacterized Ax esters ( $27.1 \pm 1.1\%$ ) while Ax and Cx accounted for a reduced proportion of the carotenoid pool. Carotenoid profiles were consistent among experimental cohorts with the exception of Cx present at a significantly higher level in nuptial male dorsally ( $5.7 \pm 1.1\%$ ) when compared to any other cohort ( $1.0 \pm 0.2\%$ ) and ventrally ( $2.7 \pm 1.2\%$ ) when compared to silver males ( $0.7 \pm 0.1\%$ ). The proportion of Cx in nuptial male was also significantly higher in their dorsal than ventral area (Table 3) and in their red marks ( $19.7 \pm 0.8\%$ ; data not shown). Flesh and gonad carotenoids were also analyzed. Flesh carotenoid concentration was significantly lower in nuptial than in silver and intermediary fish (Male:  $-2.8 \text{ mg kg}^{-1}$ ; - 54.6%; female:  $-2.7 \text{ mg kg}^{-1}$ ; - 44.9%). This was concomitant with an altered carotenoid profile in the form of a reduced Ax and increased Cx level in nuptial fish (Table 3). Carotenoid concentrations were significantly reduced in mature gonads but, having a developed GSI at this stage, total gonad carotenoid content was 20.7 fold higher in nuptial female ( $111.3 \mu\text{g}$  against

**Table 3.** Carotenoid concentrations and profiles in the skin, flesh and gonads. Values are expressed as mean  $\pm$  SE (n = 5 fish/sex/colour group). Different superscript letters indicate significant differences between experimental groups. Italics denote significant differences between body areas within each experimental group for each parameter (ANOVA, P < 0.05). Note: Testes of silver and intermediary male were pooled per colour group and could not be statistically analyzed. Ax, astaxanthin; Cx, canthaxanthin, D, dorsal skin; V, ventral skin.

		Female			Male		
		Silver	Intermediary	Nuptial	Silver	Intermediary	Nuptial
<b>• Total carotenoid concentration</b>							
Dorsal skin	( $\mu\text{g cm}^{-2}$ )	3.3 $\pm$ 0.1 bc	3.2 $\pm$ 0.3 bc	4.7 $\pm$ 0.2 ab	3.0 $\pm$ 0.3 c	3.1 $\pm$ 0.3 c	5.6 $\pm$ 0.5 a
Ventral skin	( $\mu\text{g cm}^{-2}$ )	<i>1.8 <math>\pm</math> 0.1 a</i>	<i>1.8 <math>\pm</math> 0.1 a</i>	<i>2.1 <math>\pm</math> 0.2 a</i>	<i>1.7 <math>\pm</math> 0.2 a</i>	<i>1.7 <math>\pm</math> 0.2 a</i>	<i>3.3 <math>\pm</math> 0.4 b</i>
D/V		1.8 $\pm$ 0.2	1.9 $\pm$ 0.3	2.3 $\pm$ 0.3	1.8 $\pm$ 0.1	1.8 $\pm$ 0.1	1.8 $\pm$ 0.1
Flesh	(mg kg <sup>-1</sup> )	6.0 $\pm$ 0.2 a	5.9 $\pm$ 0.4 a	3.3 $\pm$ 0.6 bc	4.7 $\pm$ 0.1 ab	5.7 $\pm$ 0.2 a	2.4 $\pm$ 0.3 c
Gonads	(mg kg <sup>-1</sup> )	32.4 $\pm$ 2.7 a	36.8 $\pm$ 2.4 a	12.2 $\pm$ 0.3 b	3.2	2.9	2.0 $\pm$ 0.1 c
<b>• Carotenoid profile (%)</b>							
Dorsal skin	$\beta$ -Carotene	73.7 $\pm$ 2.8	72.4 $\pm$ 1.6	67.1 $\pm$ 3.8	72.3 $\pm$ 1.3	68.6 $\pm$ 3.7	65.1 $\pm$ 1.6
	Ax esters	24.0 $\pm$ 2.6	25.5 $\pm$ 1.7	30.7 $\pm$ 3.9	25.3 $\pm$ 1.4	29.4 $\pm$ 3.9	27.7 $\pm$ 1.0
	Cx	0.5 $\pm$ 0.0 a	1.1 $\pm$ 0.1 a	1.7 $\pm$ 0.4 a	0.7 $\pm$ 0.2 a	0.9 $\pm$ 0.2 a	5.7 $\pm$ 1.1 b
	Ax	0.5 $\pm$ 0.2	0.5 $\pm$ 0.0	0.3 $\pm$ 0.1	0.7 $\pm$ 0.2	0.8 $\pm$ 0.3	0.5 $\pm$ 0.1
	Unidentified	1.3 $\pm$ 0.3	0.6 $\pm$ 0.1	0.2 $\pm$ 0.0	1.0 $\pm$ 0.4	0.3 $\pm$ 0.2	1.0 $\pm$ 0.2
Ventral skin	$\beta$ -Carotene	67.4 $\pm$ 3.9	71.3 $\pm$ 2.5	69.9 $\pm$ 1.6	64.4 $\pm$ 2.3	63.3 $\pm$ 7.1	63.1 $\pm$ 3.0
	Ax esters	28.1 $\pm$ 4.1	25.3 $\pm$ 2.6	27.7 $\pm$ 1.5	30.4 $\pm$ 2.7	32.7 $\pm$ 7.9	31.9 $\pm$ 3.5
	Cx	1.1 $\pm$ 0.2 ab	1.2 $\pm$ 0.2 ab	1.4 $\pm$ 0.1 ab	0.7 $\pm$ 0.1 a	1.3 $\pm$ 0.2 ab	2.7 $\pm$ 1.2 b
	Ax	1.0 $\pm$ 0.3	0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	1.6 $\pm$ 0.4	2.1 $\pm$ 0.9	0.8 $\pm$ 0.1
	Unidentified	2.5 $\pm$ 0.4	1.3 $\pm$ 0.4	0.2 $\pm$ 0.0	2.9 $\pm$ 0.8	0.8 $\pm$ 0.6	1.5 $\pm$ 0.4
Flesh	Ax	61.4 $\pm$ 1.0 a	59.3 $\pm$ 1.1 a	50.0 $\pm$ 1.0 b	61.5 $\pm$ 0.7 a	61.5 $\pm$ 1.3 a	40.4 $\pm$ 2.0 c
	Cx	38.6 $\pm$ 1.0 a	40.7 $\pm$ 1.1 a	50.0 $\pm$ 1.0 b	38.5 $\pm$ 0.7 a	38.5 $\pm$ 1.3 a	59.6 $\pm$ 2.0 c
Gonad	$\beta$ -Carotene	1.9 $\pm$ 0.3 a	1.8 $\pm$ 0.3 a	5.9 $\pm$ 1.0 b	21.6	20.5	11.1 $\pm$ 2.4 c
	Ax esters	3.7 $\pm$ 0.8 a	4.3 $\pm$ 0.9 a	6.5 $\pm$ 1.0 a	49.4	44.5	40.5 $\pm$ 2.4 b
	Cx	53.3 $\pm$ 4.2 a	57.3 $\pm$ 2.1 a	43.8 $\pm$ 0.9 b	16.5	17.0	24.8 $\pm$ 1.5 c
	Ax	33.2 $\pm$ 3.6 a	29.2 $\pm$ 2.7 a	35.0 $\pm$ 0.6 a	11.6	16.2	21.4 $\pm$ 2.1 b
	Unidentified	3.9 $\pm$ 1.2 a	3.7 $\pm$ 0.9 a	4.4 $\pm$ 1.4 a	0.9	1.8	2.1 $\pm$ 0.1 b

5.4  $\mu\text{g}$  in silver and intermediary ovaries) and 27.2 fold higher in nuptial male (2.9  $\mu\text{g}$  against 0.1  $\mu\text{g}$  in silver and intermediary testes) (Data not shown). The prime ovarian carotenoid compound was always Cx followed by Ax with, at the mature stage, a reduction in the proportion of Cx and an increased level of  $\beta$ -carotene. Significantly different, the testicular carotenoid pool was dominated by Ax esters with a higher proportion of  $\beta$ -carotene and lower levels of Ax and Cx.

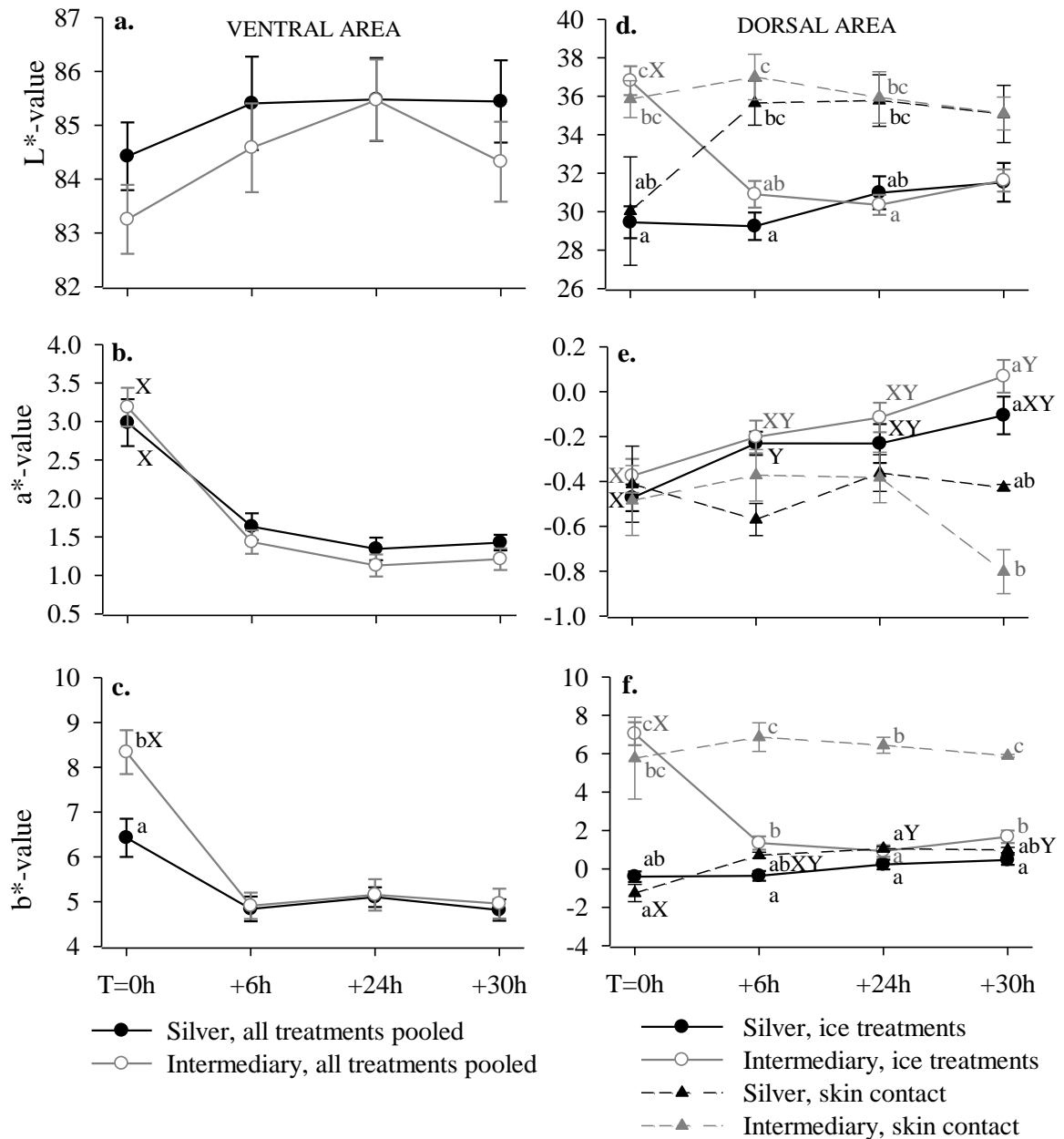
### 3.3. Post-mortem skin color reconditioning

Within each skin color category and body area, there were no significant differences in tristimulus  $L^*a^*b^*$  parameters between on-coming treatment (T0). Datasets were pooled at that time point to establish that, before treatment application, intermediary fish had a significantly higher  $b^*$ -value in both body areas and a higher  $L^*$ -value dorsally than silver fish (Table 4). Color parameters of the ventral skin varied in the same fashion regardless of the treatment and of the skin color group with a systematic reduction in both  $a^*$  and  $b^*$ -values observed from T0 to T1. The drop in  $b^*$ -value was particularly marked in intermediary fish such that no differences between cohorts remained from T1 onward in the ventral area (Fig.4a-4c). All ice treatments had the same effects on  $L^*a^*b^*$  parameters of the dorsal area and they were pooled before analysis (Fig.4d-4f). In direct ice contact, intermediary fish underwent a significant decrease in  $L^*$ -parameter from T0 to T1 to values measured in silver fish. This parameter was thereafter stable and never different between color groups. Similarly, the  $b^*$ -value of intermediary but not silver fish decreased significantly from T0 to T1 but remained significantly higher than in silver fish at T1 and T3. Finally, both color categories underwent a significant and parallel increase in  $a^*$ -value. Skin contact had a distinct effect on skin color composition. In particular  $L^*$  and  $b^*$ -values of intermediary

fish remained unchanged but L\*-parameter of silver fish increased from T0 to T1 to values measured in the intermediary cohort. This parameter did not differ between color classes but between treatments at T1 and T2. The b\*-value of silver fish in contact with skin increased from T0 to T3 but always remained significantly lower than in intermediary fish under the same conditions and never different to both color categories exposed to ice contact. Finally, the a\*-parameter, which did not vary between color groups at T0, was significantly lower at T3 in intermediary fish in contact with skin than in both color classes stored on ice.

**Table 4.** Tristimulus colour composition of silver and intermediary phenotype in the dorsal and ventral skin areas before post-mortem treatment (T0). Values are expressed as mean  $\pm$  SE (n = 30 sample/colour group from 5 fish/colour group). Different superscript letters indicate significant differences between colour group; analyzed separately for each body area (ANOVA, P < 0.05). L\*, Lightness; a\*, red-greenness; b\*, yellow-blueness.

	Dorsal skin		Ventral skin	
	Silver	Intermediary	Silver	Intermediary
L*	29.5 $\pm$ 0.8 a	36.7 $\pm$ 0.7 b	84.4 $\pm$ 0.6	83.3 $\pm$ 0.6
a*	- 0.5 $\pm$ 0.1	- 0.4 $\pm$ 0.1	3.0 $\pm$ 0.3	3.2 $\pm$ 0.3
b*	- 0.5 $\pm$ 0.3 a	6.9 $\pm$ 0.6 b	6.4 $\pm$ 0.4 z	8.3 $\pm$ 0.5 y



**Figure 4.** Tristimulus colour parameters ( $L^*$ : lightness;  $a^*$ : red-greenness;  $b^*$ : yellow-blueness) of silver and intermediary fish in the ventral and dorsal areas before treatment ( $T = 0h$ ) and following 6h ( $T_1$ ), 24h ( $T_2$ ) and 30h ( $T_3$ ) of treatment (Ice treatments: Freshwater (FW); FW + 0.25M  $Na^+$ ; FW + 0.5M  $Na^+$ ; FW + 0.75M  $Na^+$ ; FW + 5mM caffeine and direct contact with skin). Values are expressed as mean  $\pm$  SE ( $n = 5$  samples/treatment/colour group except direct skin contact where  $n = 4$  samples/colour group). Different superscript letters indicate significant differences between skin colour group within each sampling point. Different capital letters indicate significant differences between sampling points within each treatment and colour group (ANOVA,  $P < 0.05$ ).

## 4. Discussion

Significant skin color variations occur in immature Atlantic salmon that do not reflect deteriorations in flesh quality and can be minimized by direct ice contact. The seasonal occurrence and flesh quality of the three observed phenotypes (silver, intermediary and nuptial) are first discussed in relation to sexual maturation. Their pigmentary origin is then described with emphasis on carotenoid dynamics toward the expression of a sexually dichromatic nuptial color and on the involvement of purine compounds. Finally, the possibility to improve the appearance of immature Atlantic salmon exhibiting a distinctive non-silver phenotype at the post-harvest stage is discussed.

### 4.1. Relationship between phenotypes, sexual development and flesh quality

Initiation of gonadal development was observed in intermediary colored fish in July followed by the occurrence of nuptial fish at an advanced stage of sexual maturation from August onward. This is in agreement with the well reported window of sexual maturation in Atlantic salmon (Taranger *et al.*, 1998; Michie, 2001) and suggests that intermediary coloration in July represented the initiation of nuptial display for a majority of the stock exhibiting this phenotype. Their higher body size, flesh lipid but also carotenoid content could thus be expected as recruitment into maturation is inherent to a feeding surge defining the so-called anabolic window of Atlantic salmon maturation that is reported in late spring in Scotland (Aksnes *et al.*, 1986; Kadri *et al.*, 1996; Leclercq *et al.*, 2010a). Furthermore in a previous study, total flesh carotenoid positively correlated with body size and dietary lipid level (Torrissen *et al.*, 1989). Apparently contradictory, it is of interest to report a reduced flesh color score in July intermediary fish characterized by a higher flesh carotenoid and lipid content when

compared to silver-immature fish. Flesh lipids, particularly in the form of intermuscular fat, were previously shown to negatively affect the perceived color of salmonid fillet (Christiansen *et al.*, 1995b). Accordingly in this study, the higher flesh lipid content of July intermediary fish, although not significant, was most likely responsible for their lower visual flesh redness despite a higher flesh carotenoid concentration. Flesh color score was the only parameter negatively affected in July intermediary fish (-2.4 points at 24.1 points) but it remained above the commercially acceptable standards of 16 reported when using the Roche color card scale (Torrissen, 2000). Fish exhibiting an intermediary skin coloration were also observed from August to December, i.e. over the species reproductive window, at a rate ranging from 10.1% to 19.4%. During this period this phenotype correlated poorly with sexual recruitment which occurred only in few intermediary females ( $\leq 17\%$ ) in August and September and in both silver and intermediary females in November and December. Flesh quality showed no discrepancies between silver and intermediary cohorts from August to December. Overall, intermediary skin coloration was never associated with poor flesh quality and constituted a good indicator of recruitment into sexual maturation in July only i.e. not over the full duration of the species reproductive window. This was in contrast to nuptial coloration which was always associated with advanced sexual maturation and flesh quality deterioration as reported in previous salmonid studies (Aksnes *et al.*, 1986; Michie, 2001).

#### **4.2. Carotenoid dynamic during sexual maturation**

Sexual maturation in salmonids is concomitant with a redistribution of the flesh carotenoid pool to both the skin and gonads (Torrissen and Torrissen, 1985; Bjerkeng *et al.*, 1992; Nickell and Springate, 2001). In this study, total skin carotenoid increased in



the nuptial compared to silver phenotype by an average of 89% and 29% in the male and female respectively. This parameter appeared sexually dimorphic at the nuptial stage with a significant carotenoid excess in males ventrally (+53%) but not dorsally (+21%) in this study. No such sexual dichromatism has been previously described in Atlantic salmon unlike in other salmonids. At the nuptial stage and compared to females, male skin carotenoid levels were 91% higher in rainbow trout (*Oncorhynchus mykiss*, Walbaum; Bjerkeng *et al.*, 1992) and 42% higher in wild chum salmon (*Oncorhynchus keta*, Walbaum; Kitahara, 1983). Similar dimorphisms were also observed in Arctic charr (*Salvelinus alpinus*, L.; Bjerkeng *et al.*, 1992) where 17 $\beta$ -estradiol implants significantly increased skin carotenoid concentration. Skin carotenoid profiles were largely constant in all experimental groups being dominated by  $\beta$ -carotene, a yellow-orange metabolite of Cx (Schiedt *et al.*, 1988), followed by a variety of Ax esters. It is noteworthy that the proportion of orange Cx was significantly higher on the backs of nuptial males and preferentially accumulated in their red marks. Of note, the proportion of Cx in the flesh also increased at the nuptial stage and to a higher extent in males. Similarly in gonads, Cx was at a higher proportion in mature than in immature testes (+51%) but inversely reduced in ripe compared to immature ovaries where it remained the principal carotenoid compound. Gonad carotenoids have received much less attention in the male than in the female. They are present at a much lower concentration in testes than in ovaries at all developmental stages but total carotenoid content nonetheless increased 27 fold in testes and 21 fold in ovaries when mature.

Carotenoids have an array of beneficial properties, including acting as antioxidants, vitamin A precursors and free-radical scavengers against peroxide chain reactions of fatty acids and UV-light-induced photooxidation (Leclercq *et al.*, 2010d). They were previously shown to be a critical factor of sperm quality (Catoni *et al.*, 2008)

as shown in male three-spine stickleback (*Gasterosteus aculeatus*, L.) where functional fertility and testes antioxidant capacity significantly increased in fish fed a higher carotenoid level (Pike *et al.*, 2009).

The present results show that the dynamics of carotenoid redistribution and biotransformation during Atlantic salmon maturation are to a degree sex-specific which is likely to be due to differential sex-steroid hormone profiles at this stage. This could serve the development of gonad-specific carotenoid profiles according to gamete needs and the expression of sexual dichromatism in nuptial Atlantic salmon. In particular, our data highlight a specific role of Cx in male-signalling which could reflect milt quality towards mate-selection.

#### **4.3. Role of purine pigments in the expression of colors**

Purine and melanin are key pigments in the expression of teleost color but remain poorly characterized in adult Atlantic salmon skin. Purine concentrations were reduced by about 50% in both the dorsal and ventral regions of nuptial fish compared to their silver-immature siblings. This was expected from the visible loss of silver-coating in nuptial fish and previous work showing that skin purine concentration increases and correlates with skin reflectance during salmonid smoltification (Haner *et al.*, 1995; Vanstone and Market, 1968). Only G and H were identified as previously reported in immature Atlantic salmon using chromatography and spectrophotometry (Johnston and Eales, 1967). These are also the main purine compounds in other salmonids and teleosts (Vanstone and Market, 1968; Eales, 1969; Premdas and Eales, 1976). The reduced purine level in nuptial compared to immature integument was concomitant with a lower G/H ratio as found in parr compared to smolt where similar ratios are reported (Johnston and Eales, 1967). Although not assessed in this study, the distribution of

purine pigments within the different layers of the integument is also known to vary with life-stage in teleosts. In European eel, *Anguilla anguilla*, the transition from yellow to silver phenotype during the spawning migration arises solely from a redistribution of purines from the inner-skin to the outer-scale layer of the integument (Pankhurst and Lythgoe; 1982). Similarly in smolting Atlantic salmon, the parr becomes a silvery-parr from the accumulation of purine in the inner-skin layer then a silver smolt from purine deposition in the outer-scale layer. The decrease in purine pigment measured in this study in nuptial salmon is likely to occur preferentially in the outer-scale layer toward an apparent absence of silver material on the back and a creamy-yellow belly area. This would also further expose dermal melanophores which are located directly below the purine-based reflecting cells resulting in a darkening of nuptial salmon (Michie, 2001) without an actual increase in melanin content as measured here. Reduced purine levels would equally reveal the carotenoid-filled chromatophores present in the dermis (Leclercq *et al.*, 2010d) and dominated by the yellow-orange  $\beta$ -carotene. This would favour the expression of the carotenoid-based nuptial cover but also explain the higher skin yellowness ( $b^*$ ) measured in intermediary fish characterized by a reduced purine level (- 31%) when compared to the silver phenotype.

#### **4.4. Reconditioning of the intermediary phenotype**

Deviation from the desirable silver phenotype was confirmed instrumentally and related to altered pigment concentrations. Although the intermediary phenotype did not reflect altered flesh quality, it is likely to result in product rejection hence the desire to recondition it. The intermediary coloration remained when in contact with skin but not when stored on ice which induced a strong decrease in both skin lightness ( $L^*$ ) and yellowness ( $b^*$ ) to values measured in the silver phenotype. By contrast in silver fish,

those color parameters remained constant when stored on ice while skin lightness but not yellowness increased when in contact with skin. Such rapid color changes arose from the migration of pigment vesicles within their chromatosomes, mainly melanophores which are recognized as the prime factor of physiological color change and related alterations in teleost skin lightness (Fujii, 2000; Pavlidis *et al.*, 2008). They reflect variations in purine pigment concentrations and melanosome physiological state (aggregated or dispersed) in relation to the concomitant exposition of carotenoid compounds as previously addressed. Skin contact would induce melanosome aggregation increasing skin lightness in silver but not in intermediary fish where melanosomes were initially aggregated. Conversely, ice contact induced melanosome dispersion and darkening of intermediary fish only, highlighting that melanosomes were initially dispersed in silver fish. Colour differences between phenotypes are concealed by melanosome dispersion and revealed by melanosome aggregation (brought about by skin contact) when silver fish maintain their blueness due to the light-scattering effect of the overlaying purine structures. Storage condition had a significant impact on the external appearance of whole Atlantic salmon. Direct ice contact did not alter the desirable silver phenotype but significantly enhanced the appearance of initially pale-green intermediary fish through melanophore dispersion. Greatly improved, intermediary fish were nonetheless dull-dark dorsally compared to a metallic dark-blue in the silver phenotype from differences in purine levels.

This study improves understanding on the expression of skin color in Atlantic salmon. Results show that immature Atlantic salmon can exhibit significant variations in skin coloration that do not necessarily reflect alterations in flesh quality. Deviations from the desirable silver-phenotype originate from a lack of purine pigments revealing the carotenoid compounds dominated by the yellow-orange  $\beta$ -carotene. The distinctive

lightness and yellowness of these fish is exacerbated by aggregation of the melanophore induced by skin contact and conversely reduced by direct ice contact at a post-mortem stage. Maximizing ice contact of the final product at a post-harvest stage has the potential to improve and standardize whole-fish external coloration. This would facilitate optimum biomass quality grading during primary processing, which is also based on external skin coloration, but also consumer satisfaction for whom coloration is an important quality indicator. Purine pigments were found at the lowest level in nuptial fish and can be considered as critical for revealing the sexually dimorphic nuptial cover. Further research is required to assess the significance of Cx in male nuptial cover in relation to gamete quality, and to elucidate the environmental and physiological factors lowering purine levels in teleost skin. Skin reflectance related to purine level in adult Atlantic salmon could constitute a relevant welfare indicator related to desmoltification.

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**CHAPTER 5**  
**LIGHT-MANIPULATION**

## CHAPTER 5

### PAPER V

#### ***SHORT-COMMUNICATION***

**THE USE OF CONTINUOUS LIGHT TO SUPPRESS PRE-HARVEST SEXUAL MATURATION IN SEA-REARED ATLANTIC SALMON (*SALMO SALAR* L.) CAN BE REDUCED TO A FOUR MONTH WINDOW.**

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**Keywords:** Atlantic salmon *Salmo salar* L., photoperiod, sexual maturation, suppression, light manipulation

In Atlantic salmon *Salmo salar* L. sexual maturation is concomitant with a redistribution of the somatic resources and the development of nuptial colouration responsible for the low commercial value of mature fish (Michie, 2001). Maturing fish also exhibit an altered feeding activity (Kadri *et al.*, 1996; Kadri *et al.*, 1997a and b) and increased pathogen susceptibility (Bruno, 1989; St-Hilaire *et al.*, 1998; Currie and Woo, 2007) likely to compromise growth, health and welfare of the cohabiting immature cohort. The suppression of pre-harvest sexual maturation is therefore a priority in the salmon on-growing industry and is achieved by photoperiodic manipulation of the stock in the form of continuous artificial-light (LL) applied between the winter and summer solstice during the second year at sea. This 6-month period LL-regime is recognized as the most efficient by providing a key environmental signal that phase-advances the so-called “spring decision window” such that a reduced proportion of the stock meets the developmental/energetic thresholds required to proceed through maturation (Taranger *et al.*, 1998; Endal *et al.*, 2000; Oppedal *et al.*, 2006). Current knowledge on the photoperiodic entrainment of reproduction in Atlantic salmon suggests that terminating LL-exposure before the summer solstice could be equally efficient at suppressing sexual maturation. This study tested this hypothesis on a commercial scale with the objective of reducing energy usage and potential welfare impacts associated with the long-term use of powerful lighting systems in sea-pens (Migaud *et al.*, 2007a).

The trial was performed at a commercial Atlantic salmon sea-farm (56.41°N, 5.42°W, Marine Harvest (Scotland) L<sup>td</sup>., Scotland) stocked in April 2007 with S1 smolts held under natural light conditions (NL) until the start of the trial. On the 3<sup>rd</sup> January 2008, six cages (24x24x12m) holding one sea-winter (1-SW) Atlantic salmon (n=26,493±779fish/pen) with a mean live body-weight (BW) of 1566±24g were exposed to LL using 4 metal-halide light-units per pen (Pisces 400, BGB Engineering,



Grantham, UK) placed in a standard set-up. Three photoperiodic treatments were tested in duplicate: two cages were returned to NL on the 20<sup>th</sup> April (LL-Apr), 20<sup>th</sup> May (LL-May) and 18<sup>th</sup> June (LL-Jun). Throughout the experiment fish were fed the same commercial diet according to manufacturer recommendations (Biomar, Grangemouth, UK) with water temperature at 6m depth ranging between 7.1°C and 14.7°C. Batch sample-weights were performed monthly (n=120 fish/pen/month) to calculate specific growth rate (SGR) and feed conversion ratio (FCR) (Taylor *et al.*, 2006). On June 20<sup>th</sup>, 60 fish/cage were randomly anaesthetized and measured for BW ( $\pm 0.1$ g), fork length (FL,  $\pm 0.1$ cm) and Fulton condition factor (K) calculated as  $K = (BW \times 100) \cdot FL^{-3}$ . Blood was withdrawn for analysis of plasma testosterone (T) by radioimmunoassay with levels above 3ng.mL<sup>-1</sup> indicating recruitment into maturation (Duston and Bromage, 1987; Taranger *et al.* 1998). Within these fish, 30 fish/cage were sacrificed, sexed, gonad-weight measured (GW) ( $\pm 0.001$ g) and gonadosomatic index (GSI) calculated as  $GSI = (GW \times 100) \cdot BW^{-1}$ . Ovary samples were preserved in 10% buffered formalin for histological analysis and classified according to their leading oocyte stage using the primary yolk stage (the first stage of exogenous vitellogenesis) as an indicator of commitment toward maturation. Male maturity was determined based on the bimodal GSI frequency distribution in the population (Taranger *et al.*, 1998). Single pen harvest sampling allowed accurate estimation of maturation rate through external observation of 1000 fish/pen minimum using nuptial colouration as a reliable indicator of maturity (Leclercq *et al.*, 2010a). Additionally, a minimum of 80 apparently immature fish/pen were sexed, weight-lengthed and their sexual development determined through GSI and gonad histology. Due to commercial imperatives, one cage per treatment was harvested within a 7-day period both in October and November 2008 (Harvest group 1 and 2 respectively). BW, K and GSI were assessed in June by one-way nested ANOVA and

pooled per treatment where no significant differences occurred. The effect of treatment and time was then assessed independently for each harvest group by a two-way ANOVA manipulated by a General Linear Model. Datasets were transformed when required to meet the assumptions of normality and homogeneity of variance. A Tukey's post-hoc multiple comparisons test was applied where statistical differences occurred. Maturation rates at harvest were compared by a Chi-square test. Analyses were performed using SPSS v.15 and Minitab v.15 with a significance level of 5% ( $P < 0.05$ ). Data are expressed as mean  $\pm$  SEM.

SGR and FCR were similar among treatments and averaged  $0.36 \pm 0.02\% \cdot \text{day}^{-1}$  and  $1.21 \pm 0.05$  respectively from early January to late September. All experimental pens had the same BW and K in January (not shown) and June (Table 1). However, LL-Jun had a significantly higher BW than LL-May in October and a higher BW and K than both other treatments in November (Table 1a, 1b). Based on the low testosterone levels measured in all individuals ( $< 1 \text{ ng} \cdot \text{mL}^{-1}$ ; not shown), the absence of exogenous vitellogenesis (Fig.1) and the unimodal GSI distribution in the male cohort, none of the fish assessed in June were sexually recruited at this time. Accordingly, maturation rates at harvest were consistently low ( $< 1.2\%$ ) with no significant differences between treatments (Table 1c). In the immature cohort and for both genders, GSI-values were always significantly higher in October and November than in June (Table 1d, 1e). Differences in GSI between treatments occurred only in the female cohort and in November when it was significantly higher in LL-May and LL-Apr treatments (Table 1e). This was confirmed by ovarian histology with a higher rate of primary yolk stage observed in October and November than in June in all treatments (Fig.1). The prevalence rate of females in early exogenous vitellogenesis was circa  $2.4 \pm 0.1\%$  in all

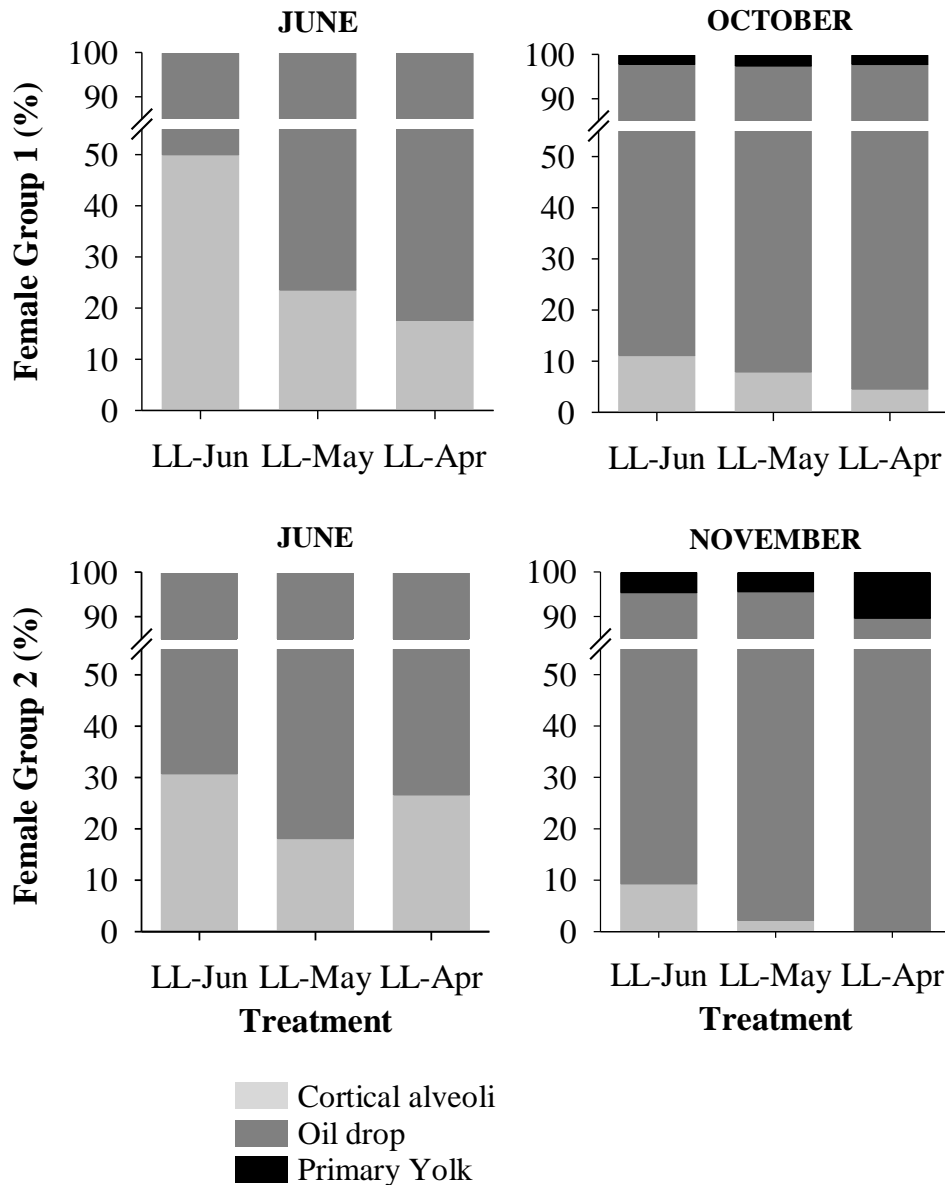
treatments in October and highest in November reaching 10.5% in LL-Apr compared to 4.7% and 4.5% in LL-Jun and LL-May respectively.

This commercial trial reports firstly that the duration of LL-exposure could be reduced without compromising its efficiency at suppressing sexual maturation and secondly the observation of females initiating exogenous vitellogenesis under a short-day photoperiod. Although no ambient unlit treatment was available as a negative control due to commercial reasons, the low maturation rates achieved are consistent with those of previous studies using LL-Jun regime (Oppedal *et al.*, 1997; Oppedal *et al.*, 2006). Our data show that LL applied during the second year at sea for 3½, 4½ and 5½ months from early January was equally efficient at suppressing the occurrence of mature salmon in autumn with no effect of the increase in ambient day-length from April to the summer solstice (LL-Apr). Previous studies have shown that the switch from short-to-long days is the key photoperiodic signal regulating Atlantic salmon maturation. An arrestment of sexual development is indeed observed within 6 weeks of LL-exposure in fish remaining subsequently immature (Taranger *et al.*, 1998, 1999; Schulz *et al.*, 2006). This photo-inhibition would have occurred before mid-April in all regimes tested here such that timings of LL-offset had no effect on maturation rates at harvest. While LL-onset rapidly photo-inhibits the immature cohort, fish undergoing further sexual development can be regarded as photo-stimulated such that Atlantic salmon populations are consistently described as sexually bimodal over long-days (Schulz *et al.*, 2006). However, it remains unclear if long-days are required for recruitment into maturation to occur. Interestingly in this study, a proportion of 2-SW females were initiating exogenous vitellogenesis in the autumn when fully mature fish also occurred. Similarly in immature 1-SW salmonids, histological and physiological evidence of sexual development was reported under short-days prior to any photoperiod

treatment (Campbell *et al.*, 2003, 2006; Taranger *et al.*, 1998). Importantly, we observed a higher proportion of true vitellogenic females in pens returned earlier to NL. This suggests that the long-to-short day switch releases the photo-inhibition of the immature cohort toward a photo-sensitive or photo-neutral stage under short-days where sexual development progress, as described in the gating model (Duston and Bromage, 1988), and actual commitment toward maturation can also occur. Exposure to LL would close this open phase within weeks by inhibiting on-going sexual development, that is to say by promoting the annual decision *not* to mature, in Atlantic salmon falling below required developmental thresholds (Thorpe, 1986; Taranger *et al.*, 1998). Importantly, stocks exposed to a shorter LL-regime had a lower harvest weight which could be expected from the stimulating effect of light on growth and appetite in salmonids (Oppedal *et al.*, 1997; Endal *et al.*, 2000; Oppedal *et al.*, 2003, 2006; Taylor *et al.*, 2006; Taylor *et al.*, 2008). Further assessment of growth and maturation of Atlantic salmon stocks exposed to the different LL-window tested is required due to genetic, environmental and husbandry variations in commercial stocks. If confirmed, the duration of LL-exposure could be significantly reduced (~35%) without compromising its efficiency at suppressing sexual maturation but should be varied according to targeted harvest BW. These preliminary results highlight the potential for reducing the duration of photoperiod manipulation, its energy usage and potential welfare impact on the stock toward a greater ecological and economic sustainability of the salmon industry.

## Acknowledgements

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**Figure 1.** Proportion of females at the different oocyte leading stages in June and October and November harvests following rearing under continuous artificial-light from early January to mid-June (LL-Jun), mid-May (LL-May) and mid-April (LL-Apr) over the second year at sea. n=15 females/pen in June; n=40 females/pen at harvest.

**Table 1** (a.) Live body-weight (BW), (b.) Fulton condition factor (K), (c.) maturation rate, (d.) male mean-GSI and (e.) female mean-GSI of Atlantic salmon *Salmo salar* L. reared under continuous artificial-light from early January to mid-June (LL-Jun), mid-May (LL-May) and mid-April (LL-Apr). Values are given as mean±SEM. Sexually recruited fish in June and fully mature fish at harvest (October and November) are not included in the dataset. Significant differences between replicates in June are shown in bold (ANOVA, P<0.05). Significant differences between treatments and month within each experimental group are shown by different superscript letter (GLM, P<0.05). Maturation rates were not significantly different (chi square test,  $\chi^2=7.782$ , P=0.169).

	June			October			November		
	LL-Jun	LL-May	LL-Apr	LL-Jun	LL-May	LL-Apr	LL-Jun	LL-May	LL-Apr
<b>(a) BW (g)</b> ( <i>n=60-80 fish/treatment/group/month</i> )									
<b>Gp 1</b>	3338±92 <sup>a</sup>	3384±125 <sup>a</sup>	3189±104 <sup>a</sup>	5139±108 <sup>c</sup>	4513±86 <sup>b</sup>	4773±81 <sup>bc</sup>			
<b>Gp 2</b>	3407±108 <sup>a</sup>	3075±104 <sup>a</sup>	3109±108 <sup>a</sup>				5529±110 <sup>c</sup>	4845±97 <sup>b</sup>	4850±93 <sup>b</sup>
<b>(b) K</b> ( <i>n=60-80 fish/treatment/group/month</i> )									
<b>Gp 1</b>	1.28±0.01 <sup>a</sup>	<b>1.26±0.02<sup>ab</sup></b>	1.25±0.02 <sup>ab</sup>	1.23±0.01 <sup>ab</sup>	1.22±0.01 <sup>b</sup>	1.23±0.01 <sup>ab</sup>			
<b>Gp 2</b>	1.24±0.01 <sup>a</sup>	<b>1.20±0.02<sup>a</sup></b>	1.19±0.02 <sup>a</sup>				1.28±0.01 <sup>b</sup>	1.21±0.01 <sup>a</sup>	1.21±0.01 <sup>a</sup>
<b>(c) Maturation rate (%) estimated based on skin colouration</b> ( <i>n=1000 fish/treatment/group/month</i> )									
<b>Gp 1</b>				0.38	0.66	0.47			
<b>Gp 2</b>							0.91	0.88	1.21
<b>(d) Male GSI (%)</b> ( <i>n=15 and n=40 fish/treatment/group/month in June and October-November respectively</i> )									
<b>Gp 1</b>	0.059±0.007 <sup>a</sup>	0.059±0.005 <sup>a</sup>	0.057±0.004 <sup>a</sup>	0.095±0.004 <sup>b</sup>	0.083±0.005 <sup>b</sup>	0.079±0.003 <sup>b</sup>			
<b>Gp 2</b>	0.058±0.003 <sup>a</sup>	0.055±0.004 <sup>a</sup>	0.049±0.004 <sup>a</sup>				0.086±0.003 <sup>b</sup>	0.078±0.002 <sup>b</sup>	0.082±0.002 <sup>b</sup>
<b>(e) Female GSI (%)</b> ( <i>n=15 and n=40 fish/treatment/group/month in June and October-November respectively</i> )									
<b>Gp 1</b>	0.202±0.013 <sup>a</sup>	0.230±0.014 <sup>a</sup>	0.249±0.048 <sup>a</sup>	0.294±0.009 <sup>b</sup>	0.294±0.001 <sup>b</sup>	0.289±0.008 <sup>b</sup>			
<b>Gp 2</b>	0.191±0.007 <sup>a</sup>	0.204±0.010 <sup>a</sup>	0.211±0.012 <sup>a</sup>				0.308±0.012 <sup>b</sup>	0.361±0.013 <sup>c</sup>	0.379±0.016 <sup>c</sup>

BW: Live body-weight, K: Fulton condition factor, GSI: gonadosomatic index; Gp1 and Gp2: Harvest group 1 and 2 respectively; LL-Jun, LL-May and LL-Apr: Artificial continuous light applied from early January to mid-June, mid-May and mid-April respectively.

## CHAPTER 5

### PAPER VI

#### **RESEARCH ARTICLE**

#### **THE POTENTIAL OF ALTERNATIVE LIGHTING-SYSTEMS TO SUPPRESS PRE-HARVEST SEXUAL MATURATION OF 1+ ATLANTIC SALMON (*SALMO SALAR*) POST-SMOLTS REARED IN COMMERCIAL SEA CAGES.**

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*Aquacultural Engineering*

**Contributions:** The present manuscript was compiled and written in full by the author of this thesis. Sampling, lab and statistical analyses have been carried out by the candidate except for trial 1 and the melatonin analyses done by Dr. Matthew Sprague. Supervisors (Drs Herve Migaud and John Taylor) provided assistance with the design of the trials, sampling and proofreading of the manuscript.

**Keywords:** *Salmo salar*, lighting-technology, light-intensity, sexual maturation, melatonin, growth.

**Abstract**

The aim of this study was to compare the efficiency of new candidate lighting-technologies (50W ‘blue’ light-emitting-diode (B,  $\lambda_{\max} = 465$  nm); 232 W ‘green’ hot cathode, (G,  $\lambda_{\max} = 546$  nm); 400 W ‘red’ tungsten-halogen, (R,  $\lambda_{\max} = 667$  to 740 nm)) against a standard 400 W ‘white’ metal-halide used as control technology (C, broad spectrum) at suppressing sexual maturation of 1+ Atlantic salmon (*Salmo salar*) in sea-cages. A total of seven experimental set-ups were tested on a commercial-scale in three trials using a standardised photoperiod regime in the form of continuous artificial-light (LL) applied from winter to summer solstice during the second year at sea. The experimental stocks were raised under an ambient thermal regime that was similar across all trials.

Technical performances (spectral output, light-attenuation and irradiance distance) of the individual light-units were measured and light-perception was assessed by quantifying plasma melatonin levels. Body-size parameters (BW, FL, K) were measured at the switch-on and turn-off of the photoperiod regimes. Maturation rates were estimated at the end of the light-treatments and at harvest. The B-unit provided the shortest effective irradiance distance (distance from the light-bulb to the minimum irradiance suppressing plasma melatonin to basal day-time level =  $0.016 \text{ W m}^{-2}$ ) but the longest relative to its energy consumption; while the G- and R-units did not offer a comparative advantage over the C-unit in that regard (B>C>G>R). Nocturnal plasma-melatonin and maturation rate decreased proportionally to the light-intensity provided using a range of technologies emitting distinct spectral profiles. Light-intensity rather than light-spectral composition appeared to be the prime parameter negatively affecting sexual maturation. Maximal suppression of maturation was observed in treatments depressing nocturnal plasma melatonin to a 1.2-fold but not to a 1.7-fold increase



compared to daytime levels, confirming that a threshold level of light-irradiance is necessary to obtain the desired effect. Results suggest that this can be achieved under standard commercial practices by applying, over the photoperiod regime presently used, continuous artificial-illumination with an (electrical) energy consumption of 0.28 Wh m<sup>-3</sup> generating a mean-irradiance of 0.012 W m<sup>-2</sup> and providing a minimum volume of effective irradiance equivalent to 12% of the rearing-environment. Such a low volume of biologically effective irradiance was likely sufficient due to the strong photic attraction already reported in Atlantic salmon. Maximal suppression of pre-harvest sexual maturation can be achieved in the Atlantic salmon on-growing industry using alternative light-technologies. Present data provides methods and threshold values favouring the implementation of photoperiod-manipulation to suppress pre-harvest maturation at the most advantageous scale and cost.

## 1. Introduction

In Atlantic salmon (*Salmo salar*) sexual maturation is concomitant with an altered feeding pattern (Kadri et al., 1996, 1997, Leclercq et al., 2010a), an increased pathogen susceptibility (St-Hilaire et al., 1998; Currie and Woo, 2007) and a deterioration of flesh and skin colour quality (Aksnes et al., 1986, Leclercq et al., 2010b). These detrimental effects can compromise the performance of the cohabiting immature cohort and reduce the volume of commercially valuable biomass. The control of pre-harvest maturation is therefore a priority in the on-growing salmon industry. This is successfully achieved on a commercial-scale by applying continuous artificial-light (LL) from the winter to the summer solstice during the second year at sea. The onset of LL in January has been shown to be the most effective at inhibiting gonadal development in fish below pre-determined developmental thresholds (Thorpe, 1994;

Taranger et al., 1998, 1999; Endal et al., 2000; Bromage et al., 2001; Leclercq et al., 2010c). This 6-month LL-window is routinely applied by the industry using powerful, wide-spectrum lighting-systems (metal-halide) which have a high-running-cost and potential welfare impacts (Migaud et al., 2007a). The industry would therefore greatly benefit from the implementation of optimized lighting-strategies that reduce operational costs and maximise the targeted biological effects, i.e. reduce maturation rate but also increase growth rates.

The annual photoperiod is widely acknowledged as the key environmental “zeitgeber” synchronizing the endogenous reproductive cycle of salmonids to the annual calendar-time (Bromage et al., 2001; Migaud et al., 2010; Taranger et al., 2010). In comparison, temperature has a minor role in the proximate control of salmonid reproductive cycles and acts as an ultimate cue synchronising, in particular, final gamete maturation and spawning (Bromage et al., 2001; Taranger et al., 2010; Pankhurst and King, 2010). The intensity (quantity) and spectral composition (quality) of incident light are key properties affecting the physiological response of teleosts with, among others, effects on growth, reproduction, behaviour and stress documented (Oppedal et al., 1997, 1999; Boeuf and Le Bail, 1999; Marchesan et al., 2005; Karakatsouli et al., 2007; Migaud et al., 2010). The effects of light-intensity have been well studied over recent years and findings clearly suggest that exposure to threshold intensity levels is required to manipulate physiological functions in various teleosts (Oppedal et al., 1997; Porter et al., 1999; Taylor et al., 2005, 2006; Migaud et al., 2006, 2010). In Atlantic salmon, exposure to LL-regimes was shown to inhibit sexual maturation and enhance growth compared to natural photoperiod (NP) and at increased rates with higher light intensities (Wallace et al., 1988; Stefansson et al., 1993; Oppedal et al., 1997; 1999). However, recent findings showed that excessively high light

intensities could induce an acute transient stress response (Wallace et al., 1988; Migaud et al., 2007a) and even retinal damage (Vera et al., 2009).

Various endocrine studies both *in-vitro* and *in-vivo* have demonstrated that synthesis of the hormone melatonin, released by the light-sensitive pineal gland, accurately reflects the prevalent photoperiod in teleosts (reviewed by Falcón et al., 2010). As such melatonin is regarded as the key time-keeping hormone that can be used as a reliable indicator of light perception as its production varies inversely with the level of light-irradiance on the pineal organ (Randall et al., 1995; Yáñez and Meissl, 1996; Falcón et al., 2010; Migaud et al., 2010). More specifically, there appear to be species-specific light-irradiance thresholds above which the circadian melatonin rhythm is suppressed to basal levels such that nocturnal artificial-light is perceived as daylight (Migaud et al., 2006). This threshold would be in the region of  $0.016 \text{ W m}^{-2}$  in Atlantic salmon (Migaud et al., 2006; Vera et al., 2010).

If many studies have focused on the effects of light-intensity, only a few have looked at the effects of light spectral composition on fish physiology. The teleost pineal gland also exhibits a spectral sensitivity to the incident light which appears to be adapted to the species natural habitat (Karakatsouli et al., 2007; Vera et al., 2010). In European sea bass (*Dicentrarchus labrax*), shorter wavelengths (blue light  $\lambda$  450 nm) were found to be the most effective at suppressing circulating melatonin levels although longer wavelengths (red light  $\lambda$  700 nm) were also potent if applied above intensity thresholds (Bayarri et al., 2002; Vera et al., 2010). Similarly, in Atlantic salmon, *in-vitro* studies showed that red light ( $\lambda$  650 nm) was less efficient at suppressing melatonin than blue ( $\lambda$  450 nm) and green ( $\lambda$  550 nm) light although data on spectral sensitivity remain scarce in this species (Migaud et al., 2010; Vera et al., 2010). In rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), blue and

red wavelengths appeared to decrease growth performance and increase stress factors in comparison to full visible spectrum from white fluorescent lamps (Karakatsouli et al., 2007; 2008).

To date, present knowledge on spectral-sensitivity and threshold light-intensity have not been applied to Atlantic salmon reared in commercial sea-cages. Narrow bandwidth lighting-systems offer the potential for tailoring the spectral output to the sensitivity of the species thereby optimizing the use of energy into generating the most suitable wavelengths (Loew and McFarland, 1990; Migaud et al., 2006). In addition to energy-savings, the biological potency of an increased range of lighting-technologies would allow selection of the most appropriate based on a variety of technical, practical and health and safety considerations. The aim of this study was to compare the efficiency of alternative lighting-technologies and assess their biological impact on 1+ Atlantic salmon sexual maturation in comparison to an industry standard lighting-system. Comparisons are made at the methodological, technical and economical levels with the view to assist selection of new candidate lighting-systems for use in the Atlantic salmon industry.

## **2. Materials and Methods**

Four submerged lighting-technologies generating distinct spectral outputs and intensities were assessed in a serie of trials. Each trial used mixed-sex 1+ post-smolt Atlantic salmon of the same strain (AquaGen AS, Trondheim, Norway) stocked in commercial sea-cages (Marine Harvest Ltd., UK) between February and April and exposed to an LL-regime from January to June during their second year at sea. The square-cages were set-up in rows of two (1.5 m to 2 m distance between cages) with, at

most, one cage-side adjacent to another experimental cage-side in order to prevent possible light pollution between cages.

## **2.1. Fish stock and rearing conditions**

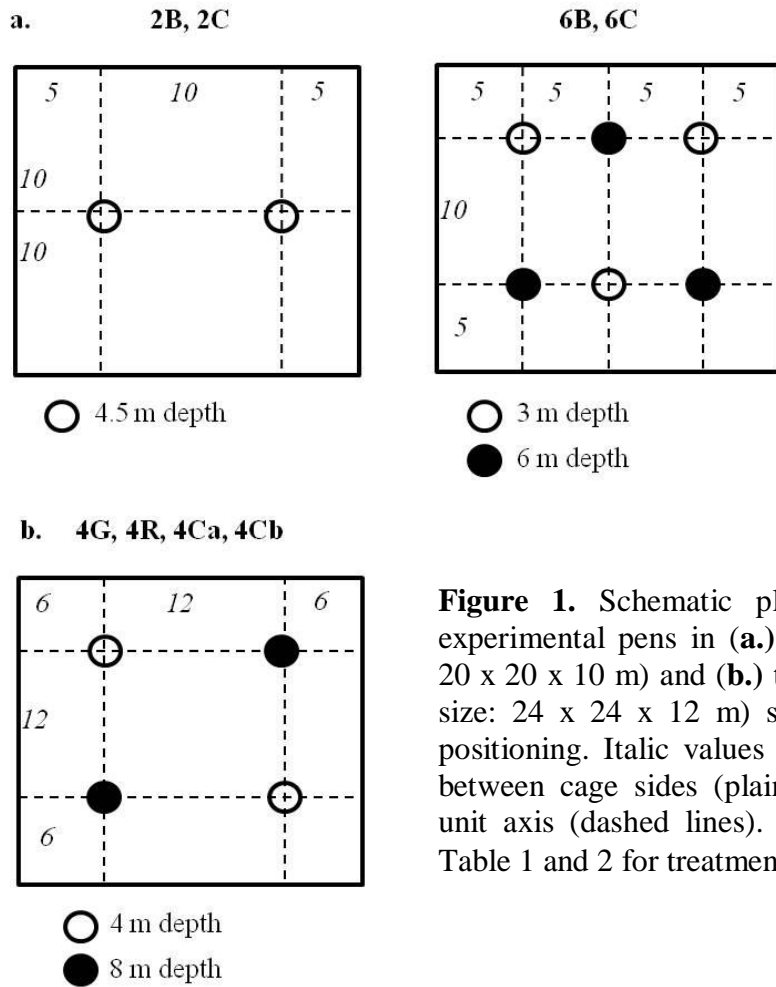
### **Trial 1**

The first trial was conducted at a commercial salmon farm (56.41°N, 5.10°W, Loch Leven; Marine Harvest Ltd., UK) using 1+ Atlantic salmon stocked at sea in March 2004. All fish were held under ambient conditions prior to the commencement of the trial. On the 15<sup>th</sup> January 2005, one sea winter (1-SW) fish with a mean live body-weight (BW) of  $1850 \pm 260$  g were distributed in six seawater cages (4000 m<sup>3</sup>; 20 x 20 x 10 m; n = 15,500-17,500 fish/pen). Duplicate unlit cages, separated from the other experimental cages by unlit (non-experimental) cages to prevent artificial-light pollution, were maintained under ambient light-conditions as natural photoperiod controls (NP; sunlight has a continuous spectrum of all visible wavelengths) and four others were subjected to continuous artificial-light (LL) from the 22<sup>nd</sup> January 2005. In those, two different light-technologies were tested: wide-spectrum 'white' metal-halide lamp as control light-technology (C, 400 W unit<sup>-1</sup>, 3700°K, Pisces 400, BGB Engineering, Grantham, UK; same technology as used in trial 2 and 3) and a narrow bandwidth 'blue' light-emitting-diode (LED) system (B, 50 W unit<sup>-1</sup>, Akvasmart UK Ltd., Inverness, UK). For each technology, two and six units per cage were installed providing a total of four LL-treatments (Metal-halide: 2C, 6C; 'Blue' LED: 2B and 6B). No replicated design could be performed in this trial except for control natural photoperiod pens (NP). Positioning of the light-units was standardised between technologies and selected in an attempt to maximize light distribution within the rearing volume: units were submerged at 4.5 m depth in cages receiving two lamps (2C and 2B;

Fig. 1a) while, in cages equipped with six units (6C and 6B), they were submerged at 3 m and 6 m depth in two inverted triangular formations (Fig. 1a). All pens were returned to NP on 5<sup>th</sup> June 2005 which was immediately followed by a three-way size grading after which treatments could not be kept discrete due to commercial requirements. Ambient water temperature ranged from 7.5°C to 13°C during the period of light-manipulation and fish were fed a commercial diet (MHS Atlantic, Skretting, UK) according to standard commercial feeding protocols.

## **Trial 2**

The second trial was conducted at a nearby commercial salmon farm (56.41°N, 5.42°W, 35 km from trial 1 sea-site, Loch Sunart; Marine Harvest Ltd., UK) using 1+ fish stocked at sea in March-April 2007. All fish were held under ambient conditions prior to the commencement of the trial. On the 3<sup>rd</sup> January 2008, four 6912 m<sup>3</sup> cages (24 x 24 x 12 m) holding 1-SW fish (n = 23,000 to 26,500 fish/pen; BW = 1631 ± 27 g) were exposed to LL using 2 different lighting-technologies in duplicate design. Two cages received 4 metal-halide units as control light-technology (4Ca; 400 W unit<sup>-1</sup>; same technology as used in trial 1 and 3) and the other two 4 narrow bandwidth 'green' hot cathode lamps (4G, 232 W unit<sup>-1</sup>, Intravision Aqua AS, Snarøya, Norway). In all treatments, light-units were positioned in a 12 x 12 m square formation and submerged to depths of 4 and 8 m (2 units/depth; Fig. 1b). The submersion depth of the G units, which were 1.80m long, refers to the middle point of the bulb. All pens were returned to NP on 18<sup>th</sup> June 2008 and experimental groups were kept discrete until harvest in October 2008. Ambient water temperature ranged from 7.1°C to 12.1°C during the period of light-manipulation and fish were fed a commercial diet (Biomar, Grangemouth, UK) according to standard commercial feeding protocols.



**Figure 1.** Schematic plan view of the experimental pens in (a.) trial 1 (pen-size: 20 x 20 x 10 m) and (b.) trial 2 and 3 (pen-size: 24 x 24 x 12 m) showing light-unit positioning. Italic values are distances (m) between cage sides (plain line) and light-unit axis (dashed lines). Not at scale, see Table 1 and 2 for treatment description.

### Trial 3

The third trial was conducted at the same site as trial 1 using 1+ fish stocked at sea in February 2008. All fish were held under ambient conditions prior to the commencement of the trial. On the 19<sup>th</sup> January 2009, four 6912 m<sup>3</sup> cages (24 x 24 x 12 m) holding 1-SW fish ( $n = 29,000$  to 35,000 fish/pen) with a BW of  $2293 \pm 95$  g were exposed to LL using 2 different lighting-technologies in duplicate design. Two cages received 4 metal-halide units as control light-technology (4Cb; 400 W unit<sup>-1</sup>; same technology as used in trial 1 and 2) and the other two cages received 4 'red' tungsten-halogen lamps (4R, 400 W unit<sup>-1</sup>, Atlantis-light, Inishowen Engineering, Shandrum, Ireland). All units were submerged to depths of 4 and 8 m as in trial 2. All pens were returned to NP on 5<sup>th</sup> June 2009 and experimental groups were kept discrete until

harvest in September 2009. Ambient water temperature ranged from 6.6°C to 13.9°C over the period of light-manipulation and fish were fed a commercial diet (Biomar, Grangemouth, UK) according to standard commercial feeding protocols.

## 2.2 Light-output and light-perception

Characteristics of the light emitted by the different technologies (B, G, R, C) were measured on the same night and sea-site (May 2010; Loch Leven) on individual light-units submerged at 3 m depth. The spectral composition (over the visible spectrum:  $\lambda$  400-740 nm) was determined using a portable spectroradiometer (StellarNet Inc. EPP2000c; Tampa, FL, USA; calibrated to National Physics Laboratory UK standards; Migaud et al., 2007b) directly pointing at the light-source and positioned 0.5 m from each unit. Horizontal profiles of light-attenuation were determined by measuring light-irradiance over the visible spectrum ( $\lambda$  400-740 nm;  $\text{W m}^{-2}$ ) using a single channel light-energy sensor pointing directly at the light-source and connected to a hand-held digital meter (Skye Instruments Ltd., Powys, UK). Measurements were made from the light-source (0 m) and then at 0.5 m horizontal increments until the detection limit of the light-energy sensor was reached ( $<0.0001 \text{ W m}^{-2}$ ). The equation of the regression curve was used to calculate the attenuation coefficients at 1m intervals within 1 to 10 m of the light-source (values were then averaged). This equation was also applied to determine the greatest distance from the light-source to an irradiance effective at suppressing plasma melatonin to day-time levels in salmonids ( $\geq 0.016 \text{ W m}^{-2}$ ; previously determined by Migaud et al., 2006). This distance was then used as the radius of a “theoretical sphere” to calculate the volume of effective irradiance emitted by point-source bulbs (B, R, C) while for light-unit G, the volume of a cylinder holding the same radius and the height of the bulb-length (1.80 m) was added. The effective irradiance



volume of individual lamps was finally used to calculate the percentage of the rearing volume theoretically subjected to an effective irradiance which assumes optimal positioning of the light-units. Using the same apparatus but with the light-energy sensor pointing upwards, vertical down-welling light-irradiance was measured in half of each treatment cage in May 2005, May 2008 and May 2009 in trial 1, 2 and 3 respectively. To do so, light-irradiance was measured in a grid format (starting in a cage corner) at 2 m horizontal increments in both horizontal directions and at 2 m (trial 1) or 1 m (trial 2 and 3) depth increments then averaged at each depth. Light-properties within the treatment pens were not measured during day-time in this study. The vertical intensity profile of artificial-illumination using C light-units in cages was previously quantified and found to be approximately 99.8% lower than natural day-light (under light-cloud conditions) (Leclercq et al., unpublished). This suggests that natural sunlight would prevail over the continuous artificial-illumination such that day-time light-conditions were consistent across all treatments. In addition, variations in ambient day-light conditions (e.g. from cloud-cover and surface-reflection) are unlikely to alter the perception of daylight as the natural photophase.

Light perception was assessed in trial 1 during both day- and night-time by measuring plasma melatonin levels at mid-day and mid-night ( $n = 20$  fish/pen) on the date of light-irradiance assessment (May 2005). Blood was withdrawn (under dim red light at night) from the caudal vein of culled fish (MS-222 bath, 150 ppm for 2 to 3 min; Alparma, Fordingbridge, England; followed by cranial percussion), centrifuged (1200 g; 15 min; 4°C) and plasma stored at -70°C until analysis using a commercially available ELISA kit (IBL, Hamburg, Germany) previously validated in salmon (Migaud et al., 2007a, 2007b). The minimum sensitivity of the kits was 3.0 pg.ml<sup>-1</sup> and the inter- and intra-assay coefficients of variation 3.8 % and 10.7 % respectively.

### 2.3 Body-size and maturational status

Body size parameters were assessed in each trial at both the onset and termination of LL-treatments in January and late May-June respectively. Fish were randomly sampled, anaesthetized (MS-222 bath, 30 ppm for 2 to 3 min) and individually measured for BW ( $\pm 5$ g) and fork length (FL,  $\pm 1$ mm) (trial 1: 225 fish/pen on the 31<sup>st</sup> January and 25<sup>th</sup> May 2005, replicated photoperiod control only; trial 2: 60 fish/pen on the 10<sup>th</sup> January and 20<sup>th</sup> June 2008, replicated treatments; trial 3: 120 fish/pen on the 20<sup>th</sup> January by batch sample-weight and 20 fish/pen on the 11<sup>th</sup> June 2009, replicated treatments). Fulton condition factor (K) was calculated as  $K = (BW \times 100) / FL^3$ . The relative weight gain (RWG; %) over the period was calculated as  $RWG = [(BW_f / BW_i) \times 100]$ ; where  $BW_f$  and  $BW_i$  are the mean final and initial BW respectively. The specific growth rate (SGR; % day<sup>-1</sup>) was calculated using  $SGR = [\exp(g) - 1] \times 100$ ; where  $g = (\ln BW_f - \ln BW_i) / (t_f - t_i)$ ,  $BW_f$  and  $BW_i$  are the same parameters as for RWG calculation and  $(t_f - t_i)$  is expressed in days.

Sexual maturation was assessed on the day of body-size assessment in late May-June when recruitment into sexual maturation is determined in Atlantic salmon populations of the Northern hemisphere (Taranger et al., 1999; Leclercq et al., 2010a). Fish were sacrificed, sexed and gonad-weight measured (GW;  $\pm 0.001$ g) to calculate the gonadosomatic index (GSI) as  $GSI = (GW / BW) \times 100$  ( $n = 25, 30$  and  $20$  fish/pen in trial 1, 2 and 3 respectively). Ovary samples were preserved in 10% buffered formalin for histological analysis and classified according to their leading oocyte stage using the primary yolk stage (the first stage of exogenous vitellogenesis) as an indicator of commitment toward maturation (Taranger et al., 1999). Males were classified as immature or sexually recruited based on the bimodal GSI frequency distribution in the

population with a threshold value of  $GSI = 0.2\%$  (Kadri et al., 1997; Taranger et al., 1998; Leclercq et al., 2010a). In addition, blood was withdrawn from randomly selected fish, centrifuged (1200 g; 15 min; 4°C) and plasma stored at -70°C for analysis of testosterone (T) level. Plasma T was analysed using an indirect competitive radioimmunoassay method (modified from Duston and Bromage, 1987) with levels above 3 ng ml<sup>-1</sup> indicating recruitment into maturation (Taranger et al., 1998). Minimum sensitivity was 1.9 pgml<sup>-1</sup>, with an intra-assay coefficient of variation of 4.4% and inter-assay coefficient of variation of 9.8% (n=15). In trial 1, plasma was sampled during a three-way body-size grading performed in June four days after gonad sampling (n = 50, 25 and 25 fish/grade/pen in the large, medium and small grade respectively) to assess the efficacy of top-crop harvest at selectively harvesting a high proportion of maturing fish. In trial 2 and 3, plasma was sampled on the day of gonad sampling (n = 60 or 20 fish/pen respectively) and maturation rate was further estimated at harvest using nuptial skin colouration as a reliable indicator (n > 1000 observations/pen in October 2008 and September 2009 respectively; Leclercq et al., 2010a and b).

## 2.4 Statistical analysis

Linear regressions were performed using GraphPad InStat between the energy consumption of the experimental set-ups and the mean light-irradiance in the sea-cage, between both those parameters and the maturation rate observed and between the cost of electricity and the value of the biomass sexually inhibited. Linear regressions always conformed to a linear model with slopes significantly different to 0. Analyses of variance in body-size and maturation parameters were performed using Minitab v.15 statistical software package. Data sets were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test, examination of residual plots), log or square-root transformed when required and proportions arc-sin transformed. Replicate

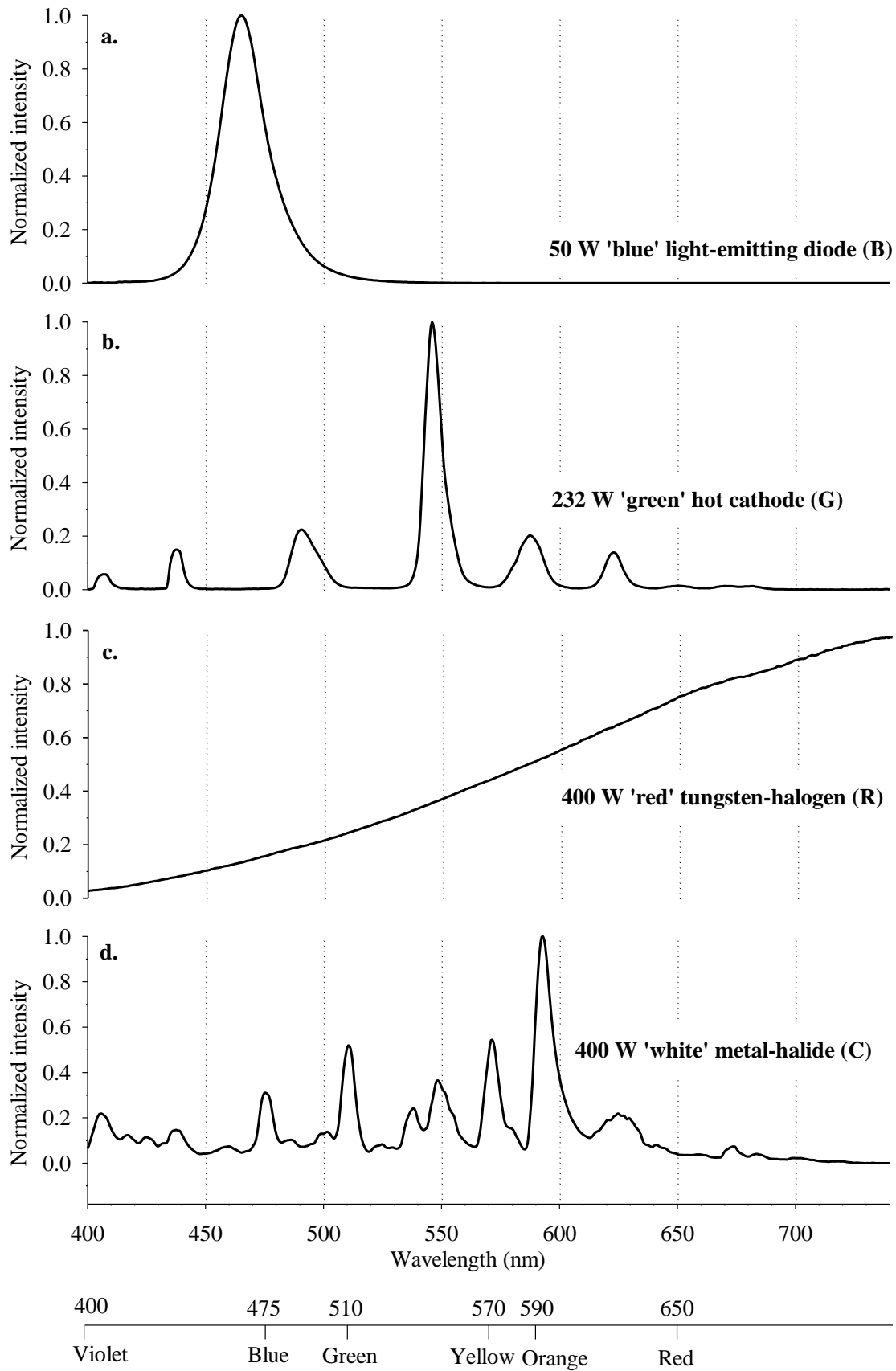
data (NP photoperiod control in trial 1; all treatments in trial 2 and 3) were pooled when no significant differences occurred. Differences in BW, FL, K, GSI and plasma-T between treatments within each trial were determined by one-way analysis of variance (ANOVA). In trial 3, two-way analysis of variance was used to test the effect of size-grade and light-treatment on plasma-T levels. Analysis of plasma-T variance systematically included BW as covariate (ANCOVA) which always had a significant effect except in trial 3 ( $p = 0.083$ ). Where statistical differences were found, *post-hoc* multiple comparisons were applied (Tukey's test; Zar, 1999). A statistical significance of  $p < 0.05$  was applied to all statistical tests. All data are presented as mean  $\pm$  S.E.M.

### 3. Results

#### 3.1. Light-output

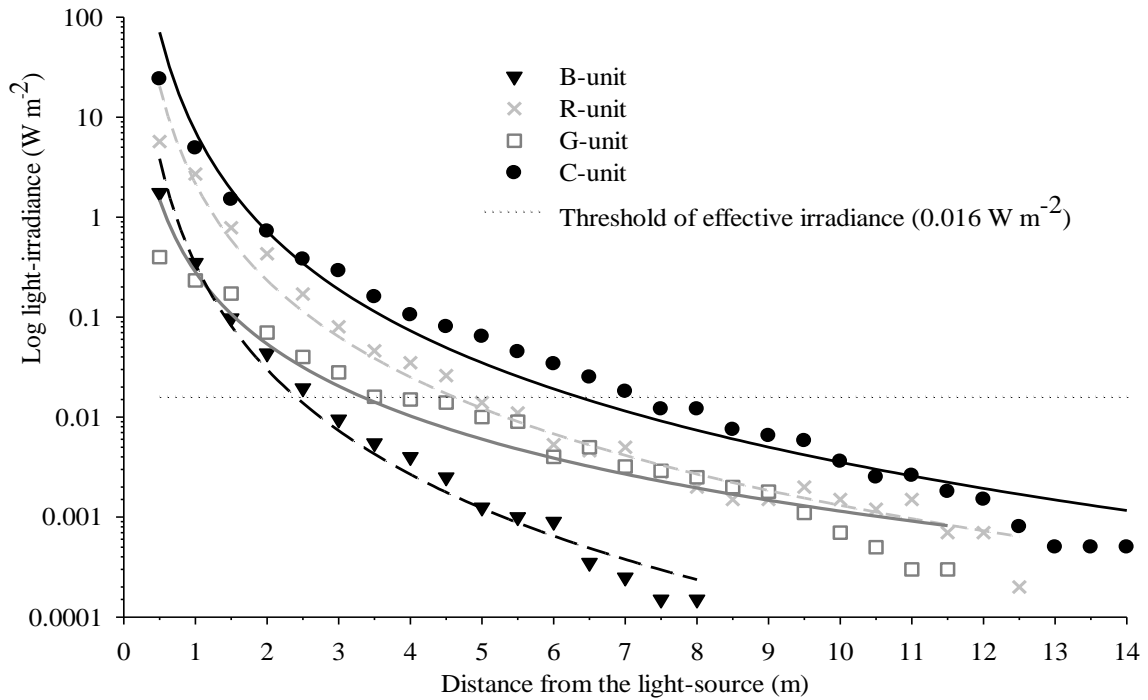
Light emitted by the four submerged technologies tested in the marine environment displayed distinct spectral profiles over the visible spectrum (Fig. 2).

The 50 W 'blue' LED unit (B) generated a single peak at  $\lambda$  465 nm, corresponding to the visible blue wavelengths, and the 232 W 'green' hot cathode (G) a main peak at  $\lambda$  546 nm within the green wavelengths. Both B and G light-units can be considered as narrow bandwidth lighting-systems. In comparison, the spectral composition of the 400 W 'red' tungsten-halogen lamp (R) progressively increased from the blue to the red end of the visible spectrum: Normalized intensity level reached 50% at  $\lambda$  586 nm (orange) and 80% at  $\lambda$  667 nm within the visible red. Finally, the 400 W 'white' metal-halide control technology (C) generated a number of peaks over 30% of normalized intensity: at  $\lambda$  475 nm (blue/cyan; 31%),  $\lambda$  511 nm (green; 51%) and  $\lambda$  571 nm (visible yellow; 54%) while the main peak was at  $\lambda$  593 nm (yellow/orange; 100%).



**Figure 2.** Spectral composition of the light emitted by the different lighting-technologies.

Horizontal profiles of visible light-attenuation were also different between lighting-technologies (Fig. 3). Irradiance ( $\text{W m}^{-2}$ ) measured at 0.5 m from the source was lowest for G, followed by B, R and highest for C. The mean light-attenuation coefficient, directly related to the power of the regression curve, was higher for B ( $52.2 \pm 4.5\% \text{ m}^{-1}$ ) and lowest for G ( $40.9 \pm 4.3 \text{ m}^{-1}$ ; Table 1). The distance from the light-source to an irradiance of  $0.016 \text{ W m}^{-2}$ , previously determined as the minimum light-intensity threshold suppressing plasma melatonin to day-time level (effective irradiance distance; Migaud et al., 2006) was longest for the C-unit (6.3 m; 100%) and comparatively reduced for all alternative technologies tested: R (4.6 m; 72.6%), G (3.3 m; 52.5%) and B (2.4 m, 37.8%; Table 1). The different systems also showed variation in their efficiency at converting energy-input into light-output. This is highlighted by the ratio of effective distance relative to the lamp energy use ( $\text{m Wh}^{-1}$ ;  $B > C > G > R$ ; data not shown) which was highest for B despite the higher attenuation of B-light in the aquatic environment. The volumes of effective irradiance emitted by the alternative light-units were always below 40% that of the C-unit (C:  $1065 \text{ m}^3$ , 100% > R:  $407 \text{ m}^3$ , 38.2% > G:  $216 \text{ m}^3$ , 20.3% > B:  $58 \text{ m}^3$ , 5.4%; Table 1). Adjusted to the number of lamps deployed in the experimental sea-cages, the theoretical volumes of effective irradiance varied between treatments (Table 2). In trial 1, it covered 100% and 53% of the rearing volume in 6C and 2C respectively but less than 10% in both B-treatments.



**Figure 3.** Horizontal profile of light-attenuation as generated by the different lighting-technologies assessed in the same environment over a single nocturnal sampling (See Table 1 for light-unit description). Equations of the respective light-attenuation curves were calculated by least-square regression (B:  $y = 0.34x^{-3.50}$ ,  $r^2 = 0.980$ ; G:  $y = 0.28x^{-2.39}$ ,  $r^2 = 0.927$ ; R:  $y = 2.18x^{-3.22}$ ,  $r^2 = 0.973$ ; C:  $y = 7.14x^{-3.31}$ ,  $r^2 = 0.965$  where  $x$  = distance from the light-source (m) and  $y$  = light-irradiance ( $W m^{-2}$ ).

**Table 1:** Parameters of light-irradiance generated by the different lighting-technologies (B: 50 W ‘blue’ light-emitting diode; G: 232 W ‘green’ hot cathode; R: 400 W ‘red’ tungsten halogen; C: 400 W ‘white’ metal-halide).

Light <sup>1</sup>	Mean attenuation coefficient <sup>2</sup> (% m <sup>-1</sup> )	Greatest distance (m) from the source to an irradiance <sup>2</sup> :		Volume (m <sup>3</sup> ) of irradiance <sup>5</sup> :	
		Effective <sup>3</sup>	Detectable <sup>4</sup>	Effective	Detectable
B (58 Wh)	52.2±4.5	2.40	8.0	58	2145
G (232 Wh)	40.9±4.3	3.32	11.5	216	7118
R (398 Wh)	49.7±4.5	4.60	12.5	407	8181
C (460 Wh)	50.5±4.5	6.34	14	1065	11494

<sup>1</sup> Electrical energy consumption of light-units (Wh) provided by light-manufacturers. <sup>2</sup> The regression curves (Fig. 3) were used to calculate the mean attenuation coefficients within 1 to 10 m of the light-source and <sup>3</sup> determine the greatest distance from the light-source to a theoretical irradiance threshold shown to suppress plasma melatonin to basal day-time levels (Effective irradiance  $\geq 0.016 W m^{-2}$ ; Migaud et al., 2006). <sup>4</sup> The minimum irradiance instrumentally detectable was  $0.0001 W m^{-2}$ . <sup>5</sup> Those distances were used to determine the corresponding volumes of irradiance.

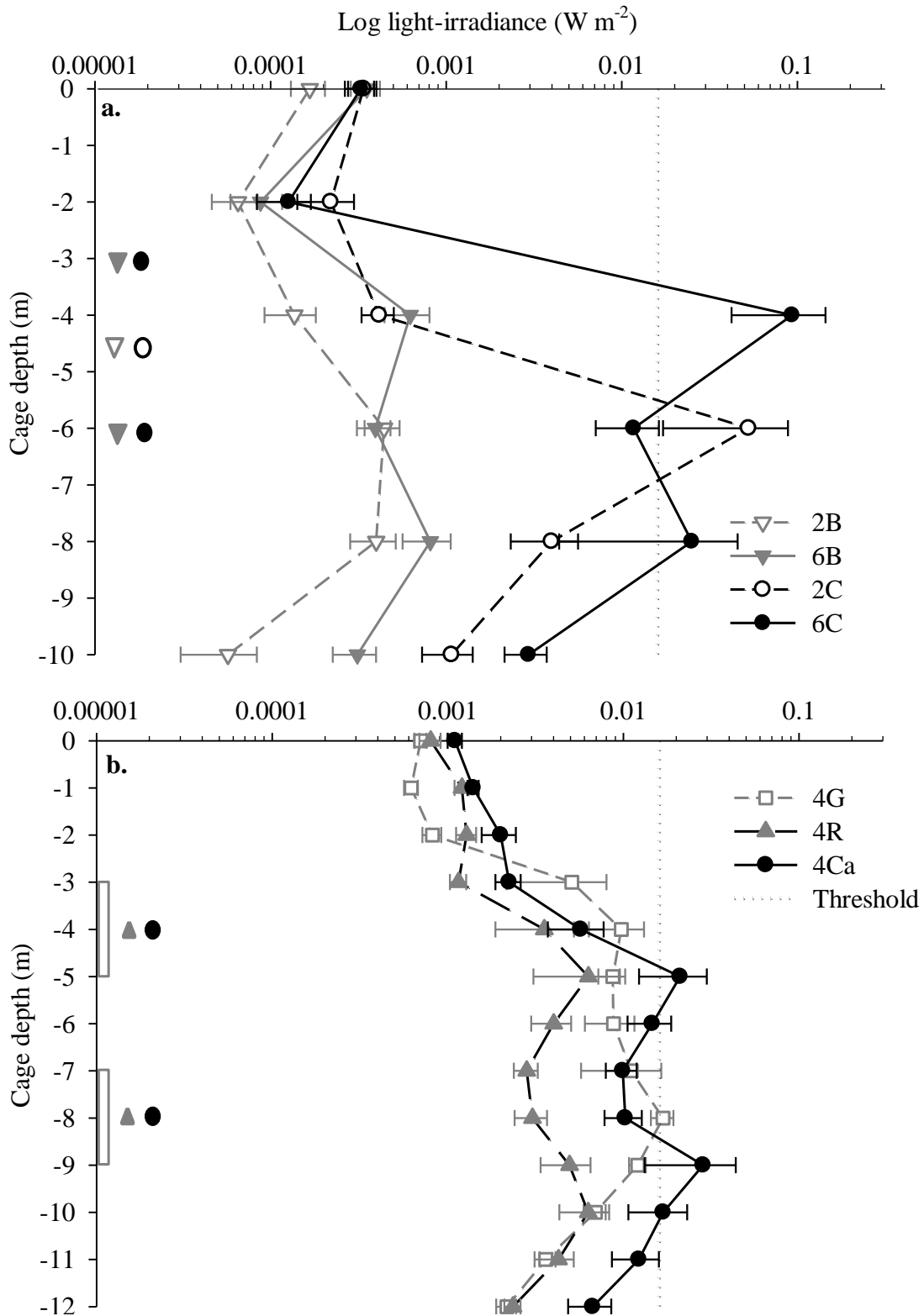
**Table 2:** Theoretical volume and proportion of the experimental cages subjected to a biologically effective irradiance level ( $\geq 0.016 \text{ W m}^{-2}$ ). Fish were reared under natural photoperiod and under 2 or 6 B or C light-units (trial 1: NP, 2B, 6B, 2C and 6C respectively) or under 4 G and 4 C light-units (trial 2: 4G and 4Ca) or under 4 R and 4 C light-units (trial 3: 4R and 4Cb). See Table 1 for description of light-units.

Treatment	Effective irradiance in the rearing-volume	
	Volume <sup>1</sup> (m <sup>3</sup> )	Proportion (%)
<b>• Trial 1 (Cages volume = 4000 m<sup>3</sup>)</b>		
NP	0	0
2B	115	2.9
6B	346	8.7
2C	2130	53.3
6C	6390	100
<b>• Trials 2 and 3 (Cages volume = 6912 m<sup>3</sup>)</b>		
4G	865	12.5
4R	1630	23.6
4Ca, b	4260	61.6

<sup>1</sup> The volume of effective irradiance in the rearing-volume was calculated by multiplying the volume of effective irradiance achieved by individual light-units (Table 1) by the number of units installed in the cage which does not take into account overlapping of light.

Variations between treatments also occurred but to a lesser extent in trial 2 and 3 with a volume of effective irradiance covering 62% of the rearing volume in 4Ca and 4Cb compared to 23.6% and 12.5% for 4R and 4G respectively. Measured on-site, vertical profiles of nocturnal down-welling light-irradiance were also lowest for 2B and 6B (Fig. 4a). Their maximum mean irradiance was  $0.0004 \text{ W m}^{-2}$  and  $0.0008 \text{ W m}^{-2}$  at 6 m and 8 m depth respectively compared to  $0.0528 \text{ W m}^{-2}$  and  $0.0933 \text{ W m}^{-2}$  at 6 m and 4 m depth for 2C and 6C respectively (trial 1). In trial 2 and 3, experimental set-ups using alternative light-units showed higher vertical irradiance profiles (Fig. 4b). Maximum mean irradiance was  $0.0168 \text{ W m}^{-2}$  at 8 m depth for 4G and  $0.0063 \text{ W m}^{-2}$  at 5 m depth for 4R as compared to  $0.0284 \text{ W m}^{-2}$  at 9 m depth for 4Ca.

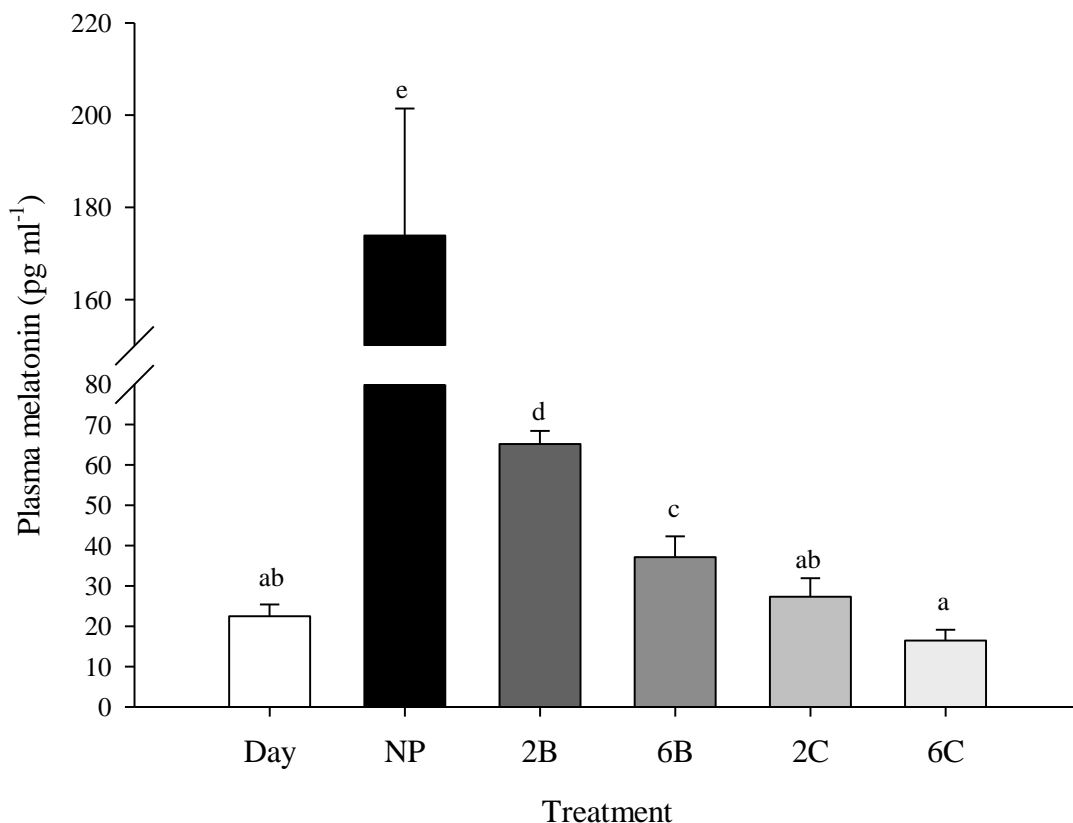




**Figure 4.** Vertical profile of nocturnal down-welling light-irradiance ( $\text{W m}^{-2}$ ; mean  $\pm$  SEM) within the experimental cages in (a.) trial 1 and (b.) trial 2 and 3 (See Table 1 and 2 for treatment description). The dashed line represents the minimum irradiance suppressing circulating plasma melatonin to basal day-time levels ( $0.016 \text{ W m}^{-2}$ ; Migaud et al., 2006).

### 3.2. Light perception: plasma melatonin levels

Day-time plasma melatonin level did not differ between treatments (data not shown) and were pooled for comparison with night-time levels (Fig. 5). At night, fish reared under LL always displayed significantly lower plasma melatonin levels than the unlit NP group which had the highest level. However, nocturnal plasma melatonin levels remained significantly higher than during the day in both B-treatments. Treatment 2B was the least effective at reducing plasma melatonin level followed by the significantly more potent 6B. Nocturnal illumination was most effective under 2C and 6C, both of which suppressed plasma melatonin to day-time levels. No such analysis could be performed in trial 2 and 3 due to the impossibility of crowding the stock within the 24 x 24 x 12m pen-systems at night.



**Figure 5.** Nocturnal plasma melatonin levels (pg ml<sup>-1</sup>) in fish exposed to different artificial-light treatments as compared to day-time levels (trial 1). Values are given as mean±SE with n = 20 fish/pen, NP in duplicate). Diurnal levels were not significantly different between treatments and therefore pooled. Different letters represent statistical differences (ANOVA,  $p < 0.05$ ; see Table 1 and 2 for treatment description).

### **3.3 Body-growth**

Before light application in January, there was no significant difference in body-size parameters between treatments within trial 2 and 3. However within trial 1, BW, FL and K were significantly higher in NP and lower in 6B (Table 3) and body-size parameters also varied between trials. The present experiment does not allow an accurate comparative assessment of the effect of light treatments on growth but data are briefly presented. Overall, body-growth parameters (RWG and SGR) appeared reduced when using alternative technologies (2B, 6B, 4G and 4R) compared to the experimental set-ups using the C-technology. Body-growth was lowest under the 2B treatment showing a RWG and SGR reduced by 38% and 32% respectively compared to C-treatments (trial 1). Both parameters were also lower in 4G compared to 4Ca (trial 2: RWG = - 4.0%; SGR = - 3.9%) and in 4R compared to 4Cb (trial 3: RWG = - 12.0%; SGR = - 9.5%).

**Table 3:** Body-size parameters between the onset (January) and the offset (June) of photoperiod manipulation using different lighting-technologies (See Table 1 and 2 for treatment description). Values are given as mean  $\pm$  SEM with  $n = 250$  fish/pen in trial 1 (NP in duplicate),  $n = 60$  fish/pen in trial 2 (duplicate treatments) and  $n = 20$  fish/pen in trial 3 (duplicate treatments). Different superscripts indicate significant differences between treatments within each trial and time point (ANOVA,  $p < 0.05$ ). Differences between replicates are shown by italic values (as measured only for K in 4Cb treatment).

		TRIAL 1					TRIAL 2		TRIAL 3	
		NP	2B	6B	2C	6C	4G	4Ca	4R	4Cb
<b>• January</b>										
BW	(g)	2233 $\pm$ 25 <sup>a</sup>	1966 $\pm$ 38 <sup>b</sup>	1560 $\pm$ 29 <sup>d</sup>	1726 $\pm$ 32 <sup>c</sup>	1759 $\pm$ 22 <sup>c</sup>	1647 $\pm$ 41	1616 $\pm$ 43	2249 $\pm$ 147	2350 $\pm$ 92
FL	(mm)	559 $\pm$ 2 <sup>a</sup>	544 $\pm$ 3 <sup>b</sup>	510 $\pm$ 3 <sup>d</sup>	526 $\pm$ 3 <sup>c</sup>	524 $\pm$ 2 <sup>c</sup>	517 $\pm$ 3	510 $\pm$ 4		
K		1.25 $\pm$ 0.01 <sup>a</sup>	1.18 $\pm$ 0.01 <sup>b</sup>	1.14 $\pm$ 0.01 <sup>c</sup>	1.15 $\pm$ 0.01 <sup>c</sup>	1.19 $\pm$ 0.00 <sup>b</sup>	1.19 $\pm$ 0.01	1.22 $\pm$ 0.01		
<b>• June</b>										
BW	(g)	3630 $\pm$ 58 <sup>a</sup>	2828 $\pm$ 62 <sup>b</sup>	2541 $\pm$ 54 <sup>c</sup>	2938 $\pm$ 54 <sup>b</sup>	3006 $\pm$ 55 <sup>b</sup>	3364 $\pm$ 73	3373 $\pm$ 71	3710 $\pm$ 157 <sup>b</sup>	4086 $\pm$ 91 <sup>a</sup>
FL	(mm)	680 $\pm$ 3 <sup>a</sup>	630 $\pm$ 4 <sup>c</sup>	619 $\pm$ 4 <sup>c</sup>	644 $\pm$ 3 <sup>b</sup>	647 $\pm$ 3 <sup>b</sup>	650 $\pm$ 4	642 $\pm$ 4	687 $\pm$ 7 <sup>b</sup>	714 $\pm$ 5 <sup>a</sup>
K		1.11 $\pm$ 0.01 <sup>a</sup>	1.09 $\pm$ 0.01 <sup>ab</sup>	1.04 $\pm$ 0.01 <sup>c</sup>	1.07 $\pm$ 0.01 <sup>bc</sup>	1.09 $\pm$ 0.01 <sup>ab</sup>	1.21 $\pm$ 0.01 <sup>b</sup>	1.26 $\pm$ 0.01 <sup>a</sup>	1.13 $\pm$ 0.04 <sup>ab</sup>	<i>1.19<math>\pm</math>0.03<sup>a</sup></i> <i>1.05<math>\pm</math>0.03<sup>b</sup></i>
<b>• Body-growth during the window of light manipulation</b>										
RWG	(%)	62.6	43.8	62.9	70.2	70.9	104.3	108.7	65.0	73.9
SGR	(% day <sup>-1</sup> )	0.43	0.32	0.43	0.47	0.47	0.51	0.53	0.38	0.42

BW: live body-weight; FL: fork-length and K: Fulton condition-factor; RWG: relative weight-gain; SGR: Specific growth-rate.

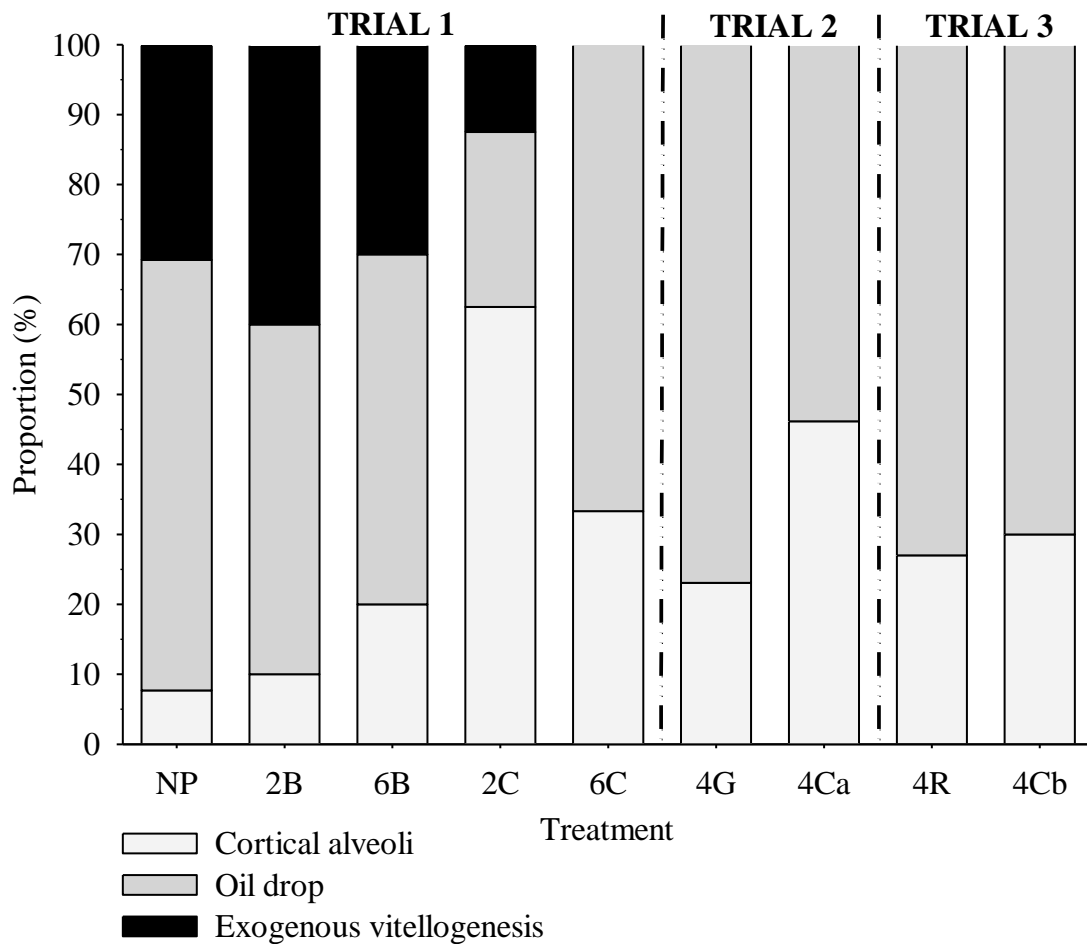
### 3.4 Sexual maturation

Following LL-application and for both genders, no significant differences in mean-GSI between treatments were observed (Table 4 with number of fish sampled). Based on the bimodal-GSI distribution in males ( $GSI > 0.2\%$ ), the occurrence of exogenous vitellogenesis in females and plasma-T levels in both genders ( $> 3 \text{ ng ml}^{-1}$ ), no randomly sampled fish were deemed to be maturing in June in trial 2 and 3. This was confirmed at harvest (September-October) by observation of skin colouration in a large random sample of the population ( $n > 1000$ ). Maturation rates were estimated below 2% in all treatments except in one replicate of treatment 4Cb (trial 3) where it reached 8.8%. In comparison, all indicators of sexual maturation showed a large variability between treatments in trial 1. None of the males displayed a GSI above 0.2% in the 2C and 6C treatments (trial 1) while around 12% were found in 2B, 6B and NP treatments. Similarly, none of the females sampled were undergoing exogenous vitellogenesis in 6C (trial 1), as observed in all treatments tested in trial 2 and 3, while it reached 12.5%, 30% and 40% in 2C, 6B-NP and 2B respectively (Table 4; Fig. 6). Although the low number of fish sampled for gonad analysis is acknowledged, plasma-T analysis overall confirmed the effect of light treatment determined from histological analysis and, in particular, the low maturation rates in trial 2 and 3 and in 2C and 6C treatments from trial 1 (Table 4). This parameter further highlighted the effect of body-size grading in June on segregating the maturing cohort within the leading body-weight cohort. As shown by the statistical differences in mean plasma-T, maturing fish ( $\text{plasma-T} > 3 \text{ ng ml}^{-1}$ ) were mainly present in the large-grade where they accounted for 45% of the fish sampled in NP compared to 30% and 26% in respectively 2B and 6B treatments. In comparison, maturation rate were always low in the medium and small size-grades ( $<4\%$ ).

**Table 4:** Indicators of sexual development at the offset of photoperiod manipulation (June) in the different experimental groups (See Table 1 and 2 for treatment description; Trial 1: duplicate photoperiod control (NP) only, Trial 2 and 3: duplicate treatments). Values are given as mean  $\pm$  SEM with number of fish assessed given in the table. Different superscripts indicate significant differences between treatments. Different letters represent significant differences between body-size grades within experimental groups (ANOVA for GSI; ANCOVA for plasma-T using BW as covariate;  $p < 0.05$ ). *Italic values correspond to maturation rates determined at harvest based on nuptial skin colouration (Rep1: replicate 1 and Rep2: replicate 2; measured in October for trial 2 and September for trial 3; n > 1000 observations/pen).*

	TRIAL 1					TRIAL 2		TRIAL 3	
	NP	2B	6B	2C	6C	4G	4Ca	4R	4Cb
• <b>Male mean GSI (%) and, in brackets, number of males assessed/treatment and proportion of males with a GSI above 0.2%</b>	0.105 $\pm$ 0.015 (23; 13.0%)	0.093 $\pm$ 0.037 (11; 12.5%)	0.075 $\pm$ 0.021 (12; 11.1%)	0.057 $\pm$ 0.007 (14; 0.0%)	0.077 $\pm$ 0.014 (13; 0.0%)	0.059 $\pm$ 0.004 (33; 0.0%)	0.058 $\pm$ 0.003 (31; 0.0%)	0.082 $\pm$ 0.004 (19; 0.0%)	0.093 $\pm$ 0.004 (22; 0.0%)
• <b>Female mean GSI (%) and, in brackets, number of females assessed/treatment and proportion of ovaries undergoing exogenous vitellogenesis</b>	0.274 $\pm$ 0.031 (27; 30.8%)	0.297 $\pm$ 0.042 (14; 40.0%)	0.247 $\pm$ 0.035 (13; 30.0%)	0.296 $\pm$ 0.062 (11; 12.5%)	0.200 $\pm$ 0.019 (12; 0.0%)	0.216 $\pm$ 0.011 (27; 0.0%)	0.197 $\pm$ 0.008 (29; 0.0%)	0.223 $\pm$ 0.007 (21; 0.0%)	0.244 $\pm$ 0.010 (18; 0.0%)
• <b>Mean plasma T level (ng ml<sup>-1</sup>) and, in brackets, number of fish assessed/treatment/grade and proportion of fish with plasma-T levels above 3 ng ml<sup>-1</sup></b>	<i>Graded population</i>					<i>Ungraded population</i>			
Large	3.24 $\pm$ 0.29 <sup>a</sup> x (100; 44.6%)	2.56 $\pm$ 0.38 <sup>a</sup> x (50; 30.0%)	1.86 $\pm$ 0.23 <sup>a</sup> (50; 26.0%)	0.76 $\pm$ 0.16 <sup>b</sup> xy (50; 4.0%)	1.68 $\pm$ 0.17 <sup>a</sup> x (50; 4.0%)				
Med.	0.90 $\pm$ 0.06 <sup>a</sup> y (50; 0.0%)	0.27 $\pm$ 0.04 <sup>a</sup> y (25; 0.0%)	1.40 $\pm$ 0.13 <sup>b</sup> (25; 4.0%)	0.24 $\pm$ 0.08 <sup>a</sup> x (25; 0.0%)	0.58 $\pm$ 0.19 <sup>a</sup> y (25; 4.0%)	0.64 $\pm$ 0.02 <sup>b</sup> (120; 0.0%)	0.60 $\pm$ 0.01 <sup>a</sup> (120; 0.0%)	0.70 $\pm$ 0.01 (40; 0.0%)	0.77 $\pm$ 0.02 (40; 0.0%)
Small	1.10 $\pm$ 0.09 <sup>bc</sup> y (50; 2.0%)	0.26 $\pm$ 0.04 <sup>ab</sup> y (25; 0.0%)	0.97 $\pm$ 0.10 <sup>c</sup> (25; 0.0%)	1.01 $\pm$ 0.23 <sup>bc</sup> y (25; 4.0%)	0.14 $\pm$ 0.08 <sup>a</sup> y (25; 0.0%)	<i>Rep 1: 0.62%</i>	<i>0.38%</i>	<i>1.79%</i>	<i>1.61%</i>
						<i>Rep 2: 1.52%</i>	<i>0.91%</i>	<i>0.00%</i>	<i>8.82%</i>

GSI: Gonadosomatic index; T: Testosterone; Med.: Medium



**Figure 6.** Proportion of females at the different oocyte leading stages in late June corresponding to the offset of continuous artificial-light (classified according to Taranger et al., 1999; see Table 1 and 2 for treatment description).

### 3.5. Cost-benefit analysis

Energy consumption, light-irradiance and inhibition of sexual maturation are presented jointly in Table 5. Experimental cages equipped with alternative lighting-systems always used less electrical energy relative to the rearing volume ( $\text{Wh m}^{-3}$ ) than the C-treatments. In trial 1, electrical consumption of 2B, 6B and 2C were 4.2%, 12.6% and 33.3% respectively of the 6C set-up. In trial 2 and 3, energy consumption of 4G and 4R were 50.4% and 86.5% of 4Ca and 4Cb respectively.

**Table 5:** (a.) Energy consumption, mean irradiance and maturation rate observed in the different experimental groups. (b.) Inhibition of maturation by light manipulation. c. Running cost-benefit analysis of the different lighting-systems tested (See Table 1 and 2 for treatment description).

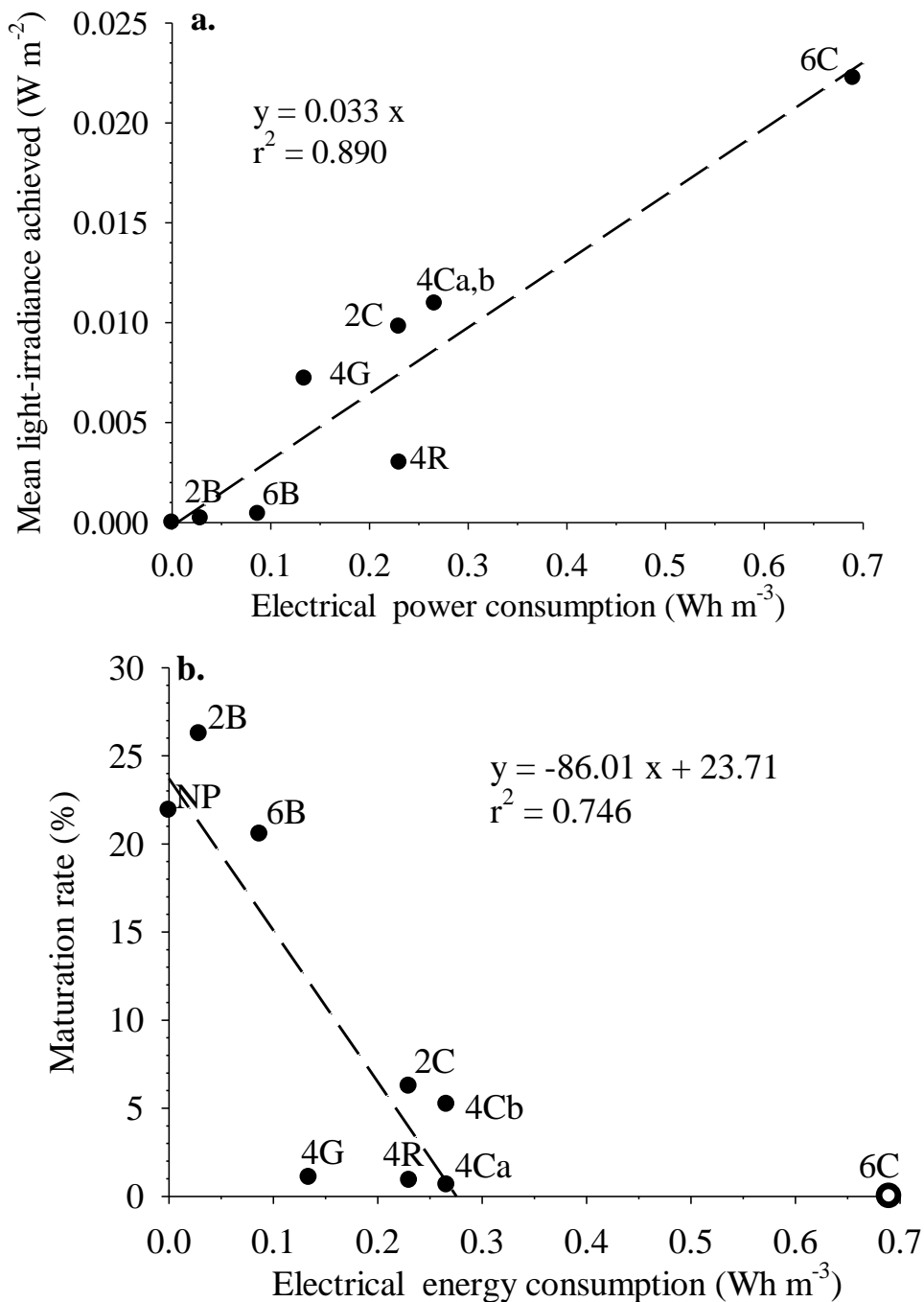
		NP	TRIAL 1				TRIAL 2		TRIAL 3	
			2B	6B	2C	6C	4G	4Ca	4R	4Cb
<b>a. Lighting-systems and maturation rate observed</b> ( <i>Cage volume = 4000 m<sup>3</sup> in trial 1 and 6913 m<sup>3</sup> in trial 2 and 3</i> )										
Electrical consumption <sup>1</sup>	(Wh light-unit <sup>-1</sup> )		58	58	460	460	232	460	398	460
	(Wh pen <sup>-1</sup> )	0	116	348	920	2760	928	1840	1592	1840
	(Wh m <sup>-3</sup> )	0	0.029	0.087	0.230	0.690	0.134	0.266	0.230	0.266
Mean irradiance	(W m <sup>-2</sup> )	0	0.0002	0.0004	0.010	0.022	0.007	0.011	0.003	0.011
Total population <sup>2</sup>	(n)	14500	14500	14500	14500	14500	25000	25000	25000	25000
Maturation rate <sup>3</sup>	(%)	21.9	26.25	20.55	6.25	0	1.07	0.65	0.9	5.2
<b>b. Inhibition of sexual maturation by light manipulation (as compared to NP)</b>										
Population sexually inhibited	(n)	0	-631	196	2269	3176	5208	5313	5250	4170
Biomass sexually inhibited <sup>4</sup>	(kg)	0	-2838	881	10212	14290	23434	23906	23625	18765
	(kg m <sup>-3</sup> )	0	-0.71	0.22	2.55	3.57	3.39	3.46	3.42	2.71
	(kg kWh <sup>-1</sup> m <sup>-3</sup> )	0	-97.9	10.1	44.4	20.7	174.6	89.8	102.6	70.5
<b>c. Running cost-benefit analysis</b>										
Total cost of electricity <sup>5</sup>	(£)	0	63	188	497	1490	501	994	860	994
	(£ m <sup>-3</sup> )	0	0.02	0.05	0.12	0.37	0.07	0.14	0.12	0.14
Value of biomass inhibited <sup>6</sup>	(£)	0	-8444	2621	30380	42512	69715	71121	70284	55826
	(£ m <sup>-3</sup> )	0	-2.11	0.66	7.59	10.63	10.08	10.29	10.17	8.08
Net saving (on electrical cost) <sup>7</sup>	(£)	0	-8507	2433	29883	41022	69214	70127	69425	54832
	(£ m <sup>-3</sup> )	0	-2.13	0.61	7.47	10.26	10.01	10.14	10.04	7.93
Economic return <sup>8</sup>		0	-134.8	13.9	61.2	28.5	139.1	71.6	81.8	56.2

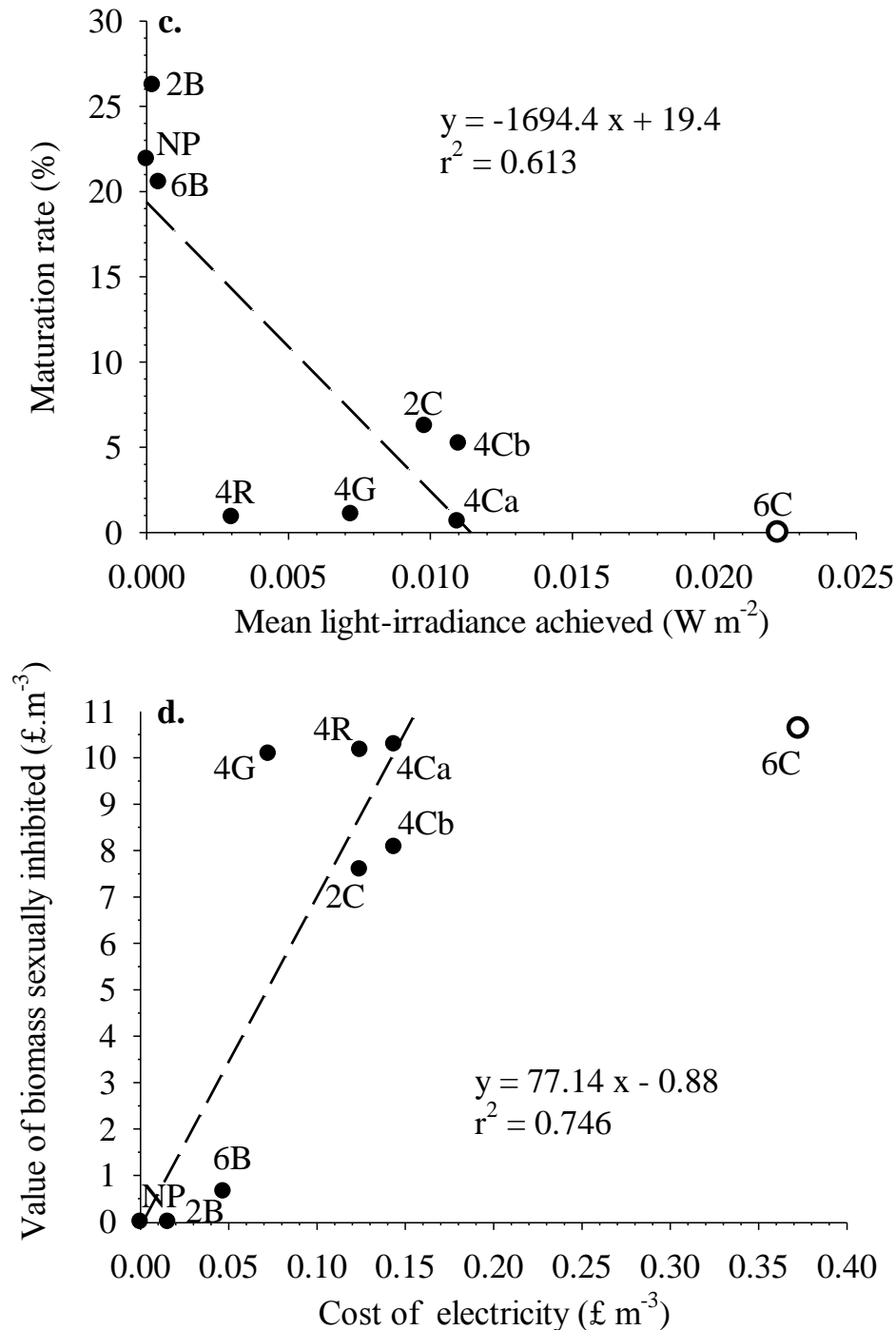
<sup>1</sup>Provided by light manufacturers. <sup>2</sup>Standardized to a constant value and density approximating experimental data for validity of the comparison. <sup>3</sup>Trial 1: Based on the proportions of male with a GSI above 0.2% and of female undergoing exogenous vitellogenesis (Table 4) that were averaged (a strictly balanced [1:1] sex-ratio was used); trial 2 and 3: using the proportion of fish exhibiting nuptial display at harvest (Table 4). <sup>4</sup>Estimated mean live-body weight at harvest: 4.5 kg. <sup>5</sup>Cost of electricity = £0.15 k Wh<sup>-1</sup> with usage of artificial-light standardized to 5 months (3600 h) in all treatments. <sup>6</sup>Gutted salmon market price = £3.5 kg<sup>-1</sup> with a whole:gutted salmon ratio of 0.85. <sup>7</sup>Calculated as: value of inhibited biomass - cost of electricity. <sup>8</sup>Calculated as: value of inhibited biomass / cost of electricity. <sup>7,8</sup> Value of mature fish (“rebate”) not considered.



Among all experimental groups, there was a significant positive linear correlation between the (electrical) energy consumption relative to the rearing volume ( $\text{Wh m}^{-3}$ ) and the mean-irradiance achieved in the sea-cage ( $\text{W m}^{-2}$ ; Fig. 7a). Of note, 4R generated the lowest mean light-irradiance relative to its energy use. Significant and negative linear correlations were measured between both the relative energy consumption or the mean-irradiance and the pen maturation rate (Fig. 7b, 7c). The 6C set-up had the highest energy use and mean-irradiance while inducing a total inhibition of maturation. The illumination provided by 6C was arguably far above the level required for maximum biological efficiency. This treatment was therefore not included in the analyses of regression between technical and biological parameters (Fig. 7b, 7c, 7d) in order to maintain the relevance of the relationships. Both regressions show that compared to 4G and 4R, all C treatments were less efficient at inhibiting sexual maturation relative to their energy use and irradiance achieved. Treatment 4G was the most effective at inhibiting sexual maturation relative to its energy use (Fig. 7b) while 4R had the lowest maturation rate relative to the irradiance emitted in the sea-cage (Fig. 7c). With virtually no effect on maturation rate, both B treatments had the highest maturation rate and lowest electrical consumption (Fig. 7b, 7c). The quantity of sexually inhibited biomass per unit of energy used and rearing volume ( $\text{kg kWh}^{-1} \text{m}^{-3}$ ; Table 5b) further highlighted the relative efficiency of the different systems. It ranged from a gain of  $175 \text{ kg kWh}^{-1} \text{m}^{-3}$  and  $103 \text{ kg kWh}^{-1} \text{m}^{-3}$  in 4G and 4R respectively to  $10 \text{ kg kWh}^{-1} \text{m}^{-3}$  in 6B to a loss of  $98 \text{ kg kWh}^{-1} \text{m}^{-3}$  in 2B. Based on our dataset, a threshold of  $0.28 \text{ Wh m}^{-3}$  generating a mean irradiance of  $0.0114 \text{ W m}^{-2}$  is required to achieve a complete inhibition of sexual maturation (Fig. 7b, 7c). Electrical consumption and inhibition of sexual maturation were further translated into economic terms using standardized population size (and density) and duration of light exposure (Table 5c). Readily

apparent was the low electrical-cost relative to the value of the biomass sexually inhibited by photoperiod-manipulation. Among all LL-treatments, the average electrical-cost was  $\text{£}0.13 \pm 0.04 \text{ m}^{-3}$  against a value of inhibited biomass of  $\text{£}5.92 \pm 1.27 \text{ m}^{-3}$  such that the average net saving on the electrical-cost was  $\text{£}5.79 \pm 1.25 \text{ m}^{-3}$ . Lighting-strategies 4G and 4R had the highest economic return (value of biomass inhibited per unit of electrical running-cost; 4G:  $139.1 > 4R > 4Ca > 2C > 4Cb > 6C > 6B, 13.9 > 2B, 0$ ; Table 5c).





**Figure 7.** Linear relationship between (a.) maturation rate and relative consumption of electrical energy, (b.) maturation rate and mean light-irradiance in the rearing volume, (c.) mean light-irradiance in the rearing volume and relative consumption of electrical energy and (d.) relative value of biomass sexually inhibited and running cost of electricity related to light-manipulation (See Table 1 and 2 for treatment description and table 5 for data calculations). Dashed lines are the linear regression given with their respective equation and regression coefficient. All regression conformed to a linear model (a:  $r^2 = 0.895$ ;  $p < 0.001$ ; b:  $r^2 = 0.778$ ;  $p < 0.005$ ; c:  $r^2 = 0.605$ ;  $p < 0.05$  and d:  $r^2 = 0.778$ ;  $p < 0.005$ ). Note: Treatment 6C was not included in the regression analysis of datasets a. b. and c. as the light-power installed (W) and energy used (Wh) was excessively high.

## 4. Discussion

### 4.1. Comparison of light-outputs between technologies and set-ups

It is established knowledge that the red-end of the visible spectrum is the most attenuated in seawater followed by violet-blue light, which is further absorbed by fine particles such as silt, while blue-green light travels the greatest distance (Lobban and Harrison, 1994; Denny, 2008). In this study, the attenuation coefficient ( $\% \text{ m}^{-1}$ ) of the narrow bandwidth green-spectrum ( $\lambda$  546 nm) G-unit was nearly 30% lower than that of the wide-spectrum C-unit. This might however also reflect a more diffuse illumination due to the length of the G-unit bulb (1.80 m long). This is further supported by the fact that light-attenuation was similar for the R- and C-units and highest for the B-unit.

The level of irradiance emitted will also vary with the energetic consumption of the unit and its efficiency at converting energy-input into light-output. Strong linear correlations were measured between the electrical consumption and the biologically effective irradiance distance of individual lamps ( $r^2 = 0.873$ , data not shown) or the mean-irradiance measured in the rearing-volume ( $r^2 = 0.890$ , Fig. 7a.). Energy consumption of the lighting-system can therefore be considered as a key factor affecting irradiance level. However, light-output relative to energy consumption also varied between technologies and was higher for the B-unit suggesting that LED technology could ultimately achieve the same effective distance that the C-unit with a lower energy input. In contrast, the G- and R-unit did not offer a comparative advantage over the C-unit in terms of effective irradiance distance per unit of energy used.

Light-unit energy consumption and effective irradiance distance are simple and practical indicators of the illumination that can be achieved. The latter can be further converted into a theoretical volume of effective irradiance which is more relevant to the three-dimensional aquatic environment. In this study, the B-, G- and R-units achieved

5.4%, 20.3% and 38.2% of the C-unit effective irradiance volume respectively such that 18.4 B-, 4.9 G- and 2.6 R- units would be theoretically needed to equal 1 C-unit. Slight-variations in the effective irradiance distance are responsible for substantial variations in the corresponding volume as the volume of a sphere is proportional to the cube of its radius. This underpins the technical advantage of increasing the power, hence effective distance of a unit instead of multiplying their number.

Discrepancies in theoretical volume of effective irradiance also occurred within the experimental set-ups and this was reflected in the vertical profiles of nocturnal down-welling irradiance. Irradiance profiles measured in the rearing pen are dependent on light-unit positioning and hence represent the true illumination perceived by the stock. This is particularly true as they are based on down-welling light-irradiance which was shown, as opposed to up-welling irradiance, to suppress circulating plasma melatonin in seabass (Bayarri et al., 2002). This is expected to remain true in most teleost species due to the anatomic localization of the pineal gland on the dorsal surface of the teleost brain. Of note, these profiles show an apparent poorer performance of the 4R compared to the 4G set-up despite the longer effective distance of the former light-unit. This is likely to be due to the design of this early prototype incorporating a detrimental bottom cap acting as a barrier to down-welling light passage. Conversely, the length of the G-unit bulb favoured a more homogenous light distribution as evident from the more consistent irradiance between different depths.

#### **4.2. Lighting-systems and suppression of maturation**

In Atlantic salmon, a significant GSI rise occurs at an advanced stage of gonadogenesis from July onward but recruitment into maturation for completion in the fall is already determined in spring/summer (Aksnes et al., 1986; Leclercq et al., 2010b). This study used three recognized indicators of maturation in June: plasma-T

levels above 3 ng ml<sup>-1</sup> (Taranger et al., 1998; Oppedal et al., 1999), the upper GSI mode in the male cohort (Kadri et al., 1997; Leclercq et al., 2010a) and the occurrence of exogenous vitellogenesis in female gonads (Taranger et al., 1998), further confirmed using nuptial colouration in autumn (Leclercq et al., 2010b). All LL-treatments in trial 2 and 3 (4G, 4R, 4Ca,b) and 6C treatment (trial 1) were highly effective at suppressing sexual maturation. This concurs with previous studies, performed under varying conditions and latitudes using different fish stocks, demonstrating the potency of the photoperiod regime used in these trials at reducing Atlantic salmon pre-harvest sexual maturation (Hansen et al., 1992; Porter et al., 1999; Taranger et al., 1998, 1999; Endal et al., 2000; Leclercq et al., 2010c). In particular, similarly low maturation rates as those shown in trials 2 and 3 were previously reported (Oppedal et al., 1997, 2006). One exception is the 8.8% maturation rate observed in one 4Cb replicate (trial 3) despite being the established technology (metal-halide) used in the salmon farming industry. This replicate also exhibited a significantly higher K in June which, at this calendar-time, can be viewed as a consequence of the anabolic effect of sexual maturation (Kadri et al., 1996, 1997, Leclercq et al., 2010a).

#### **4.3. Light-intensity threshold and suppression of maturation**

The hormone melatonin, released by the light sensitive pineal-gland, accurately reflects the prevalent photoperiod and is a reliable indicator of light perception in teleosts (Falcón et al., 2010). Of note, plasma melatonin levels hence light-perception were not significantly different between treatments during day-time (trial 1). This confirms the idea that day-time natural illumination (sunlight) prevails and conceals the artificial-illumination regardless of variations in the lighting-technologies and in environmental conditions. During night-time, all LL-treatments in trial 1 caused a

reduction in plasma-melatonin levels below that measured under NP treatment (natural darkness). The suppression of nocturnal plasma melatonin further increased with mean-irradiance measured in the sea-cage (6C>2C>6B>2B>NP). This concurs with previous *in-vitro* and tank-based studies in Atlantic salmon and European sea bass (Yáñez and Meissl, 1996; Porter et al., 2001; Bayarri et al., 2002; Migaud et al., 2006) and was not previously reported in commercial sea-cages using different lighting-technologies, spectra and set-ups. It is acknowledged that, in the present study, discrimination between the effect of light-quantity and quality was not possible as the lighting-strategies tested emitted different intensities and spectra. However, and in line with our findings on melatonin, a significant negative linear correlation was found between mean-irradiance in the rearing volume (achieved from different spectra) and maturation rate. Our data suggest that light-intensity is the main light-property affecting biological potency and that the different light-spectrum tested can achieve the desired effects at similar intensities. Artificial-light must therefore be provided at sufficient intensity in order to mask the circadian amplitude of light-intensity to a threshold value below which it is perceived as continuous and affects reproductive events (Oppedal et al., 1997, 1999; Porter et al., 1999; Kissil, et al., 2001). In mammals it is similarly assumed that a 2-fold increase in basal day-time plasma melatonin induces a physiological response (Reiter, 1988). In this study, the increase in nocturnal plasma melatonin was 2.9-fold and 1.7-fold daytime levels in 2B and 6B respectively where maturation rates were virtually unaltered compared to NP. The levels of irradiance measured in those treatments may have therefore been too low to influence the circannual entrainment of reproduction. In contrast, 2C-treatment suppressed the nocturnal rise of plasma melatonin to a non significant 1.2-fold increase above day-time levels and maturation rate was indeed significantly inhibited. Present data show that the threshold ratio of

nocturnal:diurnal plasma melatonin levels effectively suppressing sexual maturation in 1+ Atlantic salmon was below 1.65 (2B) and around 1.22 (2C), as compared to a 7.7-fold rise under natural darkness. This confirms that light-intensity *per se* plays an important role in altering the physiological response related to sexual maturation and that plasma melatonin is a reliable indicator of light perception at the population level in a commercial sea-cage environment. However, further *in vivo* studies using experimental tank-based systems are required to distinguish the effects of light-intensity and spectrum on salmonid performance.

Another important factor affecting light perception and the potency of LL-regime is the strong photic attraction of Atlantic salmon which position themselves at the depth of the submerged light-units during night-time (Juell et al., 2003; Juell and Fosseidengen, 2004). Although not assessed in this study, this is likely to explain the statistically similar plasma melatonin levels and the low maturation rates observed in 2C and 6C groups which highlight their similar potency despite variations in energy use and irradiance levels (effective volume and down-welling profiles). Across all trials, all treatments except 2B and 6B were also similarly effective at suppressing sexual maturation. From the present dataset, the threshold volume of effective irradiance to provide would be around 12% (4G) of the sea-cage. The deployment of  $0.28 \text{ Wh m}^{-3}$  of light-energy consumption generating a mean-irradiance of  $0.012 \text{ W m}^{-2}$  in the rearing volume (Fig. 7b, c) can further be considered as a safe threshold to suppress sexual maturation of 1+ Atlantic salmon to basal levels. Although not directly comparable, it is interesting to note the proximity of this mean-irradiance threshold determined under commercial conditions with the minimum level of irradiance effectively suppressing plasma melatonin to day-time level previously determined under laboratory conditions ( $0.016 \text{ W m}^{-2}$ ; Migaud et al., 2006).



#### **4.4. Lighting-system and growth**

The present data do not allow accurate characterisation of the effect of light-intensity and light-spectrum on Atlantic salmon growth. A growth enhancement (SGR and RWG) was nonetheless apparent in populations exposed to greater irradiance levels (trial 1: 6C>2C>6B>2B; trial 2: 4Ca>4G, trial 3: 4Cb>4R). This supports previous reports in salmonids where growth and appetite were shown to be stimulated proportionally to the intensity of LL provided (Oppedal et al., 1997, 2003, 2006; Endal et al., 2000; Taylor et al., 2005, 2006). However, this was not the case in trial 2 where similar growth parameters were measured in 4Ca and 4G groups despite a reduced irradiance from the latter treatment. Notwithstanding that present growth data should be interpreted with caution, this suggests a spectrum-specific stimulation of growth or a higher sensitivity of Atlantic salmon to blue-green wavelengths. Such spectral sensitivity was previously reported (Vera et al., 2010) and discussed as adaptative to the previously experienced photic environment (Lythgoe, 1980; Shand et al., 2008). The present findings, showing that different light-technologies effectively suppress sexual maturation, warrant further testing of the effect of light-property on Atlantic salmon growth. Together, this would allow identifying the most appropriate light-technologies to be used by the Atlantic salmon industry.

#### **4.5. Cost-benefit analysis**

Our preliminary cost-benefit analysis primarily highlighted the strong benefit of photoperiod manipulation inherent to the low running-cost of electricity in comparison to value of the biomass that would otherwise sexually mature. For example, the running-cost of electricity in treatment 4Ca and 4Cb (trial 2 and 3) were equal to the

value of 0.3% of the stocked biomass and the net savings on electrical cost (based on a 21.9% maturation rate observed in NP treatment) were always over £55,000 in trial 2 and 3. Although this analysis did not include other costs (capital, bulb and maintenance cost), this demonstrates that a complete suppression of maturation must be achieved through photoperiod manipulation to optimize the sustainability of the industry. With regards to the financial assessment, this also means that minor variations in maturation rate had a strong effect on the net saving on electrical cost. The economic return on light-manipulation (the value of biomass inhibited per unit of energy expenditure) is less sensitive hence more appropriate when comparing different treatments. In trial 2 and 3, this indicator was higher for 4G and 4R as these treatments achieved a similarly high inhibition of maturation with a lower energy use in comparison to 4Ca and 4Cb. Light-manipulation strategies 4G and 4R were the most cost-effective by providing the optimal level of light-intensity with biologically potent spectrums, despite the lower technical performances of units G and R (i.e. effective irradiance distance per unit of energy used). In contrast, both B-treatments showed poor economical performance due to a low suppression of sexual maturation which is likely to be due to the undersized light-power installed and irradiance achieved. However, B-units had the highest effective irradiance distance relative to energy use suggesting that they could offer the highest financial return if applied at higher intensities (e.g. >50W). Conversely, the 6C treatment successfully suppressed sexual maturation but was oversized hence its poorer economic performances.

This study demonstrated that pre-harvest sexual maturation can be efficiently suppressed using alternative lighting-technologies and with the best financial return by providing a properly scaled level of light-intensity. The optimal light-intensity is also likely to vary, to some extent, with the light-spectrum provided which requires further

experimental assessment. The possibility to choose from a wider range of lighting-technologies is of considerable advantage in itself as selecting an aquacultural lighting-technology is a trade-off between technical (e.g. consistency of output and reliability), practical (e.g. handling and maintenance), health and safety (e.g. voltage) and economic (e.g. capital and bulb cost, life-span) parameters. Extrapolation of the present results to other commercial environment must however proceed with caution due to variations in the genetic origins of the fish, environmental and husbandry conditions. The present study warrants further testing of the effect of light-property on Atlantic salmon growth and is also expected to facilitate the assessment and deployment of effective lighting-strategies in other aquacultural systems and species.

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**CHAPTER 6**  
**STERILIZATION**

## CHAPTER 6

### PAPER VII

#### **RESEARCH ARTICLE**

#### **PARR-SMOLT TRANSFORMATION IN OUT-OF-SEASON TRIPLOID ATLANTIC SALMON (*SALMO SALAR* L.)**

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**Contributions:** The present manuscript was compiled and written in full by Dr. John Taylor with the help from Dr. Herve Migaud and the candidate. The candidate has been involved in the triploidization, sampling throughout the trial (deformity assessment, smolt index), ATPase analyses and RBC measurements. Help during sampling and ATPase analysis was also provided by BS.c student Andrew Preston (IoA). Support on site (Landcatch Natural Selection) was provided by Derrick Guy.

**Keywords:** triploid, out-of-season, smoltification, photoperiod, ATPase, Atlantic salmon

**Abstract**

Production of sterile triploid Atlantic salmon (*Salmo salar*) would appear to be the best strategy to address the growing concerns on environmental impacts of escapees. However, the industry relies on a year round supply of smolts to ensure continual production and to date the production of out-of-season (S0+) triploid smolts has not been reported. The present study demonstrates that S0+ triploid smolts can be produced using an accelerated “square-wave” photoperiod (LL - LD 9.5:14.5 - LL) under ambient water temperature. Such a regime advanced the timing of smoltification by 3 months (S0+) relative to their siblings under an ambient photoperiod (S1+) as observed through body silvering, fin darkening, decrease in condition and significantly increased gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity. Importantly, triploid S0+ smolted 4 weeks earlier than their diploid siblings reflecting their increased size. In S1+ populations, no difference in smolt time was observed although triploid fish also achieved a higher weight. Furthermore, deformity prevalence was very low during the fresh ( $\leq 5\%$ ) and seawater ( $\leq 2.5\%$ ) stages within both ploidies mainly represented by spinal deformity and operculum shortening. Family effects for growth and condition were clear, however, no significant family/ploidy interactions were observed although this was based on only two full-sib crosses. These results are promising but there is still a long way to go before triploids can become a commercial reality. Further studies are required to optimize and refine husbandry protocols, define breeding strategies especially regarding family selection and overall better understand triploid salmon physiology.

## 1. Introduction

In recent years, there have been growing public and scientific concerns expressed over genetic and ecological consequences of interactions between wild and escapee farmed Atlantic salmon (*Salmo salar*) (Hindar *et al.*, 1991; McGinnity *et al.*, 1997; Castillo *et al.*, 2008). Atlantic salmon escapees from sea cages (adult) or hatcheries (juvenile), their widespread dispersal at sea and return to rivers to spawn and interference with wild stocks have been well documented in Norway (Heggberget *et al.*, 1993), Scotland (Youngson *et al.*, 1997; Martinez *et al.*, 2001), Ireland (Crozier 1993; Cotter *et al.*, 2000) and Canada (Car *et al.*, 1997; Lacroix and Stokesbury, 2004). This clearly resulted in genetic changes in wild salmon populations. The use of sterile fish in culture has thus been proposed and advocated by many as a means to minimise the potential genetic impact of cultured salmon on wild populations (ICES, 1991; NASCO, 1991). The most effective and now well established method of sterilising Atlantic salmon is through hydrostatic pressure induction of triploidy (Johnstone and Stet, 1995; O'Flynn *et al.*, 1997; Benfey, 2001), albeit that an all-female population should be utilised as triploid males can still develop gonads and sexual characteristics.

The triploid induction process and subsequent performance of fish has been comprehensively reviewed by Benfey (1999) and Piferrer *et al.* (2009). Although there are many similarities between triploid and diploid fish, basic differences exist and conflicting results in terms of performance have been obtained in salmonids and other species. Growth has been shown to be poorer (Withler *et al.*, 1995; O'Flynn *et al.*, 1997), equal to (Galbreath and Thorgaard, 1995; McGeachy *et al.*, 1995), or even better (Oppedal *et al.*, 2003) than diploid siblings. Similarly, triploid tolerance and survival under sub-optimal conditions is suggested to be poorer (Ojolick *et al.*, 1995; Hyndman *et al.*, 2003a; Atkins and Benfey, 2008), although metabolic and physiological

mechanisms and pathways do not necessarily differ (Stillwell and Benfey, 1996; Sadler *et al.*, 2000a, 2000b; Hyndman *et al.*, 2003b; Leggat *et al.*, 2006). Triploids are also often associated with greater morphological deformities, an attribute potentially detrimental to functional physiology and harvest quality (Sadler *et al.*, 2001; Kacem *et al.*, 2004; Lijalad and Powell, 2009). Furthermore, triploids have often showed greater variability both within and between families, and subsequently, numerous studies over the last decade have recommended the need to establish selection programs for successful triploid production (Bonnet *et al.*, 1999; Friars *et al.*, 2001; Cotter *et al.*, 2002; Oppedal *et al.*, 2003; Johnson *et al.*, 2004).

The ability to produce out-of-season smolts has been one of the greatest developments and assets to the salmon aquaculture industry. Under a natural photoperiod, the decision to smolt occurs under decreasing daylength in autumn with parr-smolt transformation completed on the increasing phase in spring (Duston and Saunders, 1992). Consequently, in commercial production, photoperiod and thermal regimes can be manipulated so that fish smolt out-of-season and at ages of 1 year or less (Thrush *et al.*, 1994; Duston and Saunders, 1995; Duncan and Bromage, 1998; and Duncan *et al.*, 1998). However, to date, studies in triploid Atlantic salmon have only focused on ambient (S1) smolt production (McGeachy *et al.* 1995; O'Flynn *et al.*, 1997; Benfey, 2001; Friars *et al.*, 2001; Cotter *et al.*, 2002; Oppedal *et al.*, 2003), and it has been proposed that incomplete smoltification may explain in part, poor seawater survival and performance in triploids (Boeuf *et al.*, 1994; Galbreath and Thorgaard, 1995). Certainly it is well established that in normal diploid stocks high growth rates and sufficient energy stores allow individuals to achieve the size threshold necessary for smoltification (Solbakken *et al.*, 1994; Skilbrei, 1988; Skilbrei, 1991; Handeland and Stefansson, 2001; Berrill *et al.*, 2006) and individuals that do not meet these thresholds



fail to smolt. In this context, Withler *et al.* (1995) observed a reduced rate of smolting and smaller fish at the time of smolting in accelerated underyearling (S0) triploid coho salmon (*Oncorhynchus kisutch*) although this effect was not uniform among families. This was, however, not apparent in yearling (S1) triploid coho salmon (Johnson *et al.*, 1986) suggesting that different thresholds may exist under accelerated regimes. However, this has not been studied in triploid Atlantic salmon with Jungalwalla (1991) reporting that S1 triploids did not smolt as well as diploids, while Boeuf *et al.* (1994) reported that they smolted equally well although performance was poorer. Subsequently, it has been proposed that the threshold size required for smoltification is different for triploids than in diploids, and that it may also vary with family and rearing regime (Withler *et al.*, 1998).

As out-of-season smolt production is a vital component of the sustainability of the salmon industry it is essential that knowledge of triploid production and performance under such manipulated regimes be available if farmers are to adopt this strategy. Therefore, this study aimed to examine the feasibility of producing out-of-season triploid Atlantic salmon in comparison to a natural smolt production regime, and monitor growth performance and osmotic ability during freshwater and early seawater rearing.

## **2. Materials and Methods**

### **2.1. Fish and rearing**

The experiment was carried out at Howietoun Hatchery and Niall Bromage Freshwater Research Facility (NBFRRF) in Scotland (56° N). On 10<sup>th</sup> December 2007 two randomly chosen full-sib groups of Atlantic salmon mixed sex stock were selected (Landcatch Ltd. stocks) and egg batches were each divided into two subgroups after

fertilisation (~2700 eggs/ploidy/family), one of each being subjected to a hydrostatic pressure shock (65,500 kPa for 5 min applied 30 min post fertilisation) according to Johnstone and Stet (1995) to induce triploidy. Eggs were laid down and reared separately in four 0.35m<sup>3</sup> tanks under constant darkness until yolk-sac absorption. On commencement of first feeding (14 April 2008, 800°DD) fry were exposed to constant light (LL) and provided EWOS Micro parr diet (EWOS Ltd., Bathgate, Scotland), supplied throughout 24hours using clockwork belt feeders according to manufacturer's tables. Ambient water temperature in the hatchery was 7.3°C (± 2.3°C), O<sub>2</sub> >8mgL<sup>-1</sup> and a flow rate of 10Lmin<sup>-1</sup>. On 15th May 2008 (1140°D) the four groups of parr (2 ploidy x 2 family) were transferred to NBFRR and stocked into four 0.35m<sup>3</sup> tanks prior to smoltification (~1800parr/tank). Fish were maintained under LL (0.31 W.m<sup>-2</sup>) until 29<sup>th</sup> August 2008 and fed Skretting Nutri Parr. Ambient water temperature range for NBFRR is shown in Fig.1.

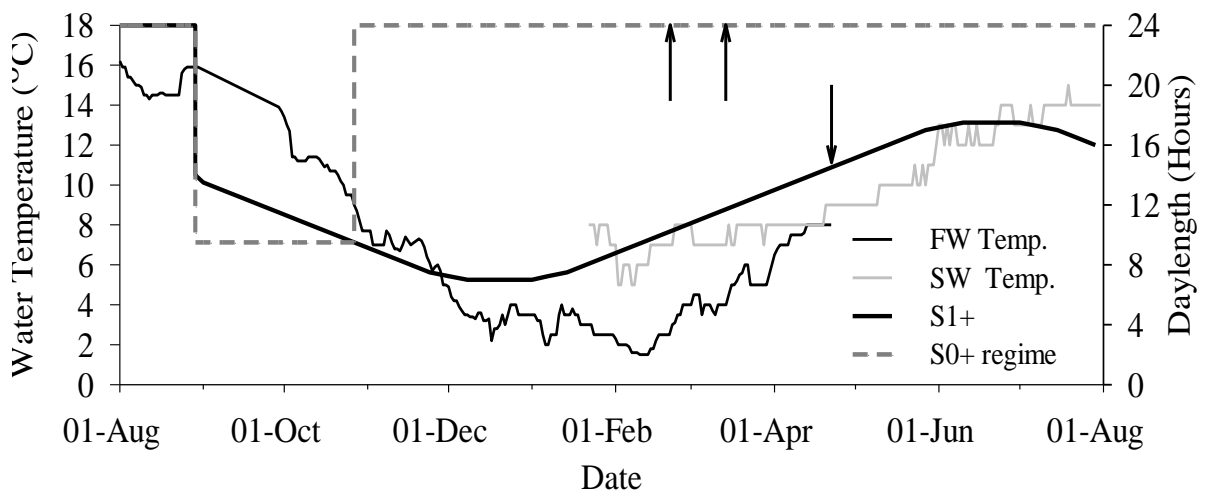
## 2.2. Smoltification regimes and saltwater transfer

On 29<sup>th</sup> August 2008 two modified photoperiod regimes were implemented to produce populations of either ambient (S1+) or out of season smolts (S0+). In each family/ploidy population 150 fish were randomly selected and placed into triplicate 0.89 m<sup>3</sup> tanks (total 12 tanks) with a flow of 10 L/min at ambient temperature (Fig.1). Potential S0+ fish were maintained on LD 9.5: 14.5 for a period of eight weeks from 29<sup>th</sup> August until 24<sup>th</sup> October, before being returned to LL until smoltification was completed. Potential S1+ fish were exposed to a simulated natural photoperiod (SNP) until smoltification was completed (Fig.1.)

Seawater transfer to Machrihanish Marine Environmental Research Laboratory (MERL, Scotland) occurred on 22<sup>nd</sup> January and 19<sup>th</sup> February 2009 for S0+ triploids

and S0+ diploids respectively. On transfer 80 fish per replicate/family/ploidy ( $n=240$ /family/ploidy) were stocked into two 1.5 m<sup>3</sup> seawater tanks (one per ploidy) with ambient temperatures (Fig.1) and flow of 20 L/min. Differentiation between the families within the communal rearing was achieved by removal of the adipose fin of family 2. Family triplicates were identified by panjet marking fish ventrally in one of three positions; anterior (rep 1), middle (rep 2) or posterior position (rep3). S0+ post-smolts were reared under LL following seawater transfer and fed Biomar Ecolife 2.0 and 3.0mm and Skretting Atlantic smolt 2.5 and 3.0mm, according to manufacturer's tables using automatic feeders throughout the normal daylight period.

S1+ diploid and triploid populations were both transferred to MERL on 19<sup>th</sup> April 2009 and stocked and marked for identification as described for S0+ smolts. S1+ post-smolts were reared under SNP following seawater transfer and fed in the same manner as S0+ post-smolts.



**Figure 1.** Photoperiod regimes used to produce an ambient S1+ smolt population (solid black) and an out-of-season S0+ smolt population (dashed grey). The ambient temperature regime of the water supply during the period of the study is also shown in both freshwater (FW; dark line) and seawater (SW; grey line). Arrows indicate seawater transfer.

## 2.3. Parameters analysed

### Ploidy determination

Blood smears were taken in both the fresh and saltwater stages (n=30/family/ploidy). Ploidy determination was assessed from blood smears prepared following severing the caudal peduncle of euthanized individuals and collecting a drop of blood. After air drying, slides were fixed in 100% methanol and then placed into Giemsa stain for 10 minutes. Erythrocyte length and diameter was measured at 400x magnification using image capture (ImagePro Software). A total of 20 randomly chosen nuclei per slide were measured to the nearest 0.01  $\mu\text{m}$ . Diploid control groups had significantly smaller RBC nuclear lengths than pressure shock triploid groups ( $P < 0.001$ ,  $2N = 7.36 \pm 0.48 \mu\text{m}$ ,  $3N = 9.64 \pm 0.54 \mu\text{m}$ ). In all samples analysed there was a 100% triploid success rate.

### Sampling protocol

During the freshwater stage, growth performance was monitored from the onset of the smolt photoperiod regimes for both (S0+) and (S1+) populations. Thereafter monthly sampling occurred, during which weight/lengths (n=20/family/ploidy/replicate), and gill ATPase (n=8-10 fish culled per tank) were collected from fish anaesthetized under 2-phenoxyethanol (0.1 ml/l). Seawater sampling was conducted two and seven days post transfer for both (S0+) and (S1+) populations (n=5 fish culled/family/ploidy/replicate), and monthly thereafter (n=30/family/ploidy). Fulton's condition factor (K) was calculated using:  $K = (WL^{-3})100$ ; where W is body weight (g) and L is fork length (mm). Sampling concluded on 31st July 2009. Specific growth rate (SGR) was calculated as  $(SGR = (e^g - 1) \times 100$ ,

where  $g = (\ln(W_2) - \ln(W_1)) \times (t_2 - t_1)^{-1}$  during the freshwater and saltwater rearing period from start point to end point based on the mean of each replicate tank.

### **Deformity prevalence**

During freshwater and saltwater sampling fish were visually inspected for morphological deformities (cataract, spinal, jaw and opercular anomalies) when growth performance data was gathered at each sampling point.

### **ATPase, Osmolality and Smolt Index**

Na<sup>+</sup>, K<sup>+</sup> - ATPase activity was determined with a kinetic assay run in 96-well microplates at 26°C and read at a wavelength of 340nm for 10 min according to the method of McCormick (1993). Protein concentrations were determined thereafter using a BCA (Bicinchoninic acid) Protein assay kit (SIGMA, Aldrich, UK).

All fish culled at each sampling point had blood removed from the caudal vein using unheparinised syringes and centrifuged at 2500 rpm for 15 min at 4 °C. Serum was collected and stored at -70 °C until analysis, when osmolality was determined using a freezing point depression osmometer (Advanced Instruments Inc., Massachusetts, USA).

Smolt index was recorded on all fish culled at each sampling point and from all fish at the time of transfer to sea using the following scale: 1, parr; 2, some silvering, parr marks visible; 3, fully silvered but, parr marks visible; 4, smolt, no parr marks visible (Sigholt *et al.*, 1995).

### **Saltwater challenge**

Seven days prior to potential seawater transfer, a total of 5 randomly selected fish / replicate / family / ploidy for both smolt populations (S0+) and (S1+) were

subjected to saltwater challenge. The test was conducted for 96 h in 50L bags of 10 °C aerated seawater at 35ppt. During the challenge the occurrence of mortalities was recorded.

## **2.4. Statistical analysis**

For each smolt regime a three-way mixed model ANOVA was performed on performance parameters (weight, K and ATPase activity) over time, considering ploidy (2N or 3N) as a fixed effect and family as a random effect, with replicate tank nested within family-ploidy grouping. Data was found to conform to normality and homogeneity of variance following Kolmogorov-Smirnov and Levene's tests and examination of residual plots. Post-hoc testing was done by Tukey's multiple comparison tests. Final performance parameters (weight, K, ATPase and SGR) at the end of the freshwater and saltwater periods were also compared between smolt regimes using a two-way ANOVA considering smolt (S0+ or S1+) and ploidy as fixed factors. Normality and homogeneity of variance were analysed as previously described. All statistical test were performed using Minitab v15 with a significance level of  $p < 0.05$ . Results are presented as mean  $\pm$  SEM.

## **3. Results**

### **3.1. Freshwater Growth and Deformity**

Mixed model ANOVA revealed a significant effect of time, ploidy and family for both weight and K in S0+ smolts during freshwater on-growing. There was a significant interaction of time with family, and time with ploidy, but no interaction of ploidy with family. A typical example of MANOVA output corresponding to weight data is shown in Table 1. In both S0+ smolt populations there was an increase in weight,

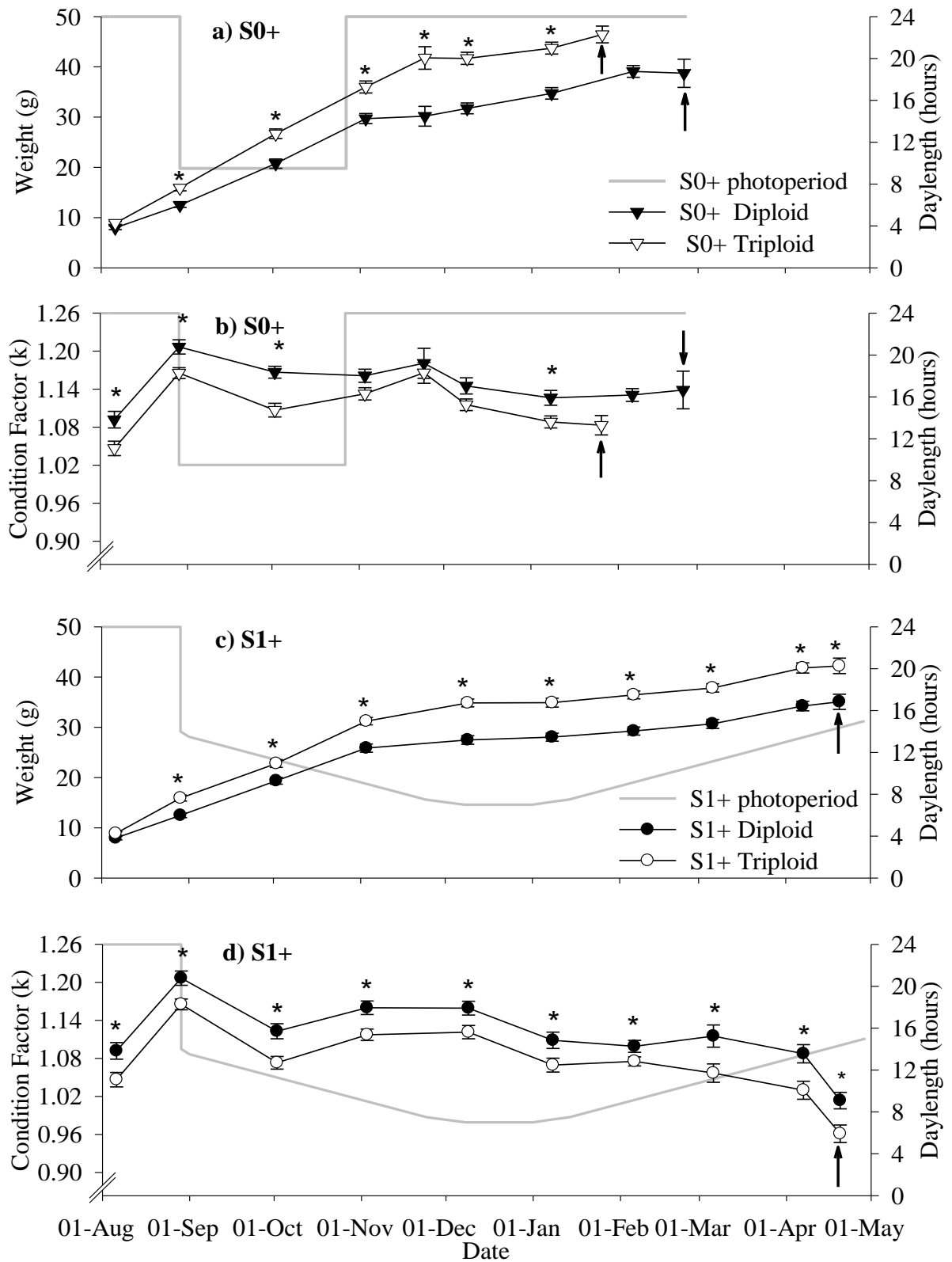
with triploids significantly heavier than diploids from the onset of the short-day winter photoperiod onwards (Fig.2a). Furthermore triploids were transferred to sea 4 weeks earlier than their S0+ diploid siblings (mid January vs. mid-February) based on the ATPase activity and smolt index score. K was significantly lower in triploid S0+ smolts at the onset of the winter short-day photoperiod, although this difference was not maintained following the return to LL in late-October (Fig.2b). In both ploidies, K decreased within 4 weeks of the return to LL and at the point of seawater transfer, K was significantly lower than at the start of the short-day photoperiod.

In the S1+ population the same interactions observed in the S0+ population were also apparent. Both ploidies showed an increase in weight with time, with triploids maintaining a significantly higher weight than their diploid siblings from the return to SNP until the time of seawater transfer in late April (Fig.2c). Triploids also exhibited a significantly lower K than diploids, with both ploidies showing a significant decrease in K during spring until the time of seawater transfer (Fig.2d).

**Table 1.** A typical example of a three-way mixed model ANOVA performed on weight data using the method of sequential sums of square for test.

Source (Weight)	df	Seq SS	Adj MS	F	P
Time	6	10459.1	1743.2	670.1	0.000
Family	1	426.1	426.1	163.8	0.000
Ploidy	1	923.6	923.6	355.1	0.000
Time x Family	6	44.9	7.49	2.9	0.018
Time x Ploidy	6	277.8	46.3	17.8	0.000
Family x Ploidy	1	2.0	2.0	0.8	0.382
Time x Family x Ploidy	6	6.2	1.03	0.4	0.878
Tank (Family Ploidy)	8	54.4	6.8	2.6	0.081
Error	48	124.9	2.6		
Total	83	12319.1			

The fixed factor within the model was ploidy (2N or 3N) with family as a random effect and replicate tank nested within family-ploidy grouping. *df*: degrees of freedom, Seq.SS: sequential sums of squares, Adj MS: adjusted sequential mean squares, F: variance and P: probability.



**Figure 2.** Changes in growth performance and condition factor (mean  $\pm$  SEM, n= 30/ploidy/family/replicate) during the freshwater phase in S0+ (a and b) and S1+ (c and d) parr for both ploidies. Arrows indicate SW transfer. Asterisk indicates significant ploidy differences ( $p < 0.05$ ).



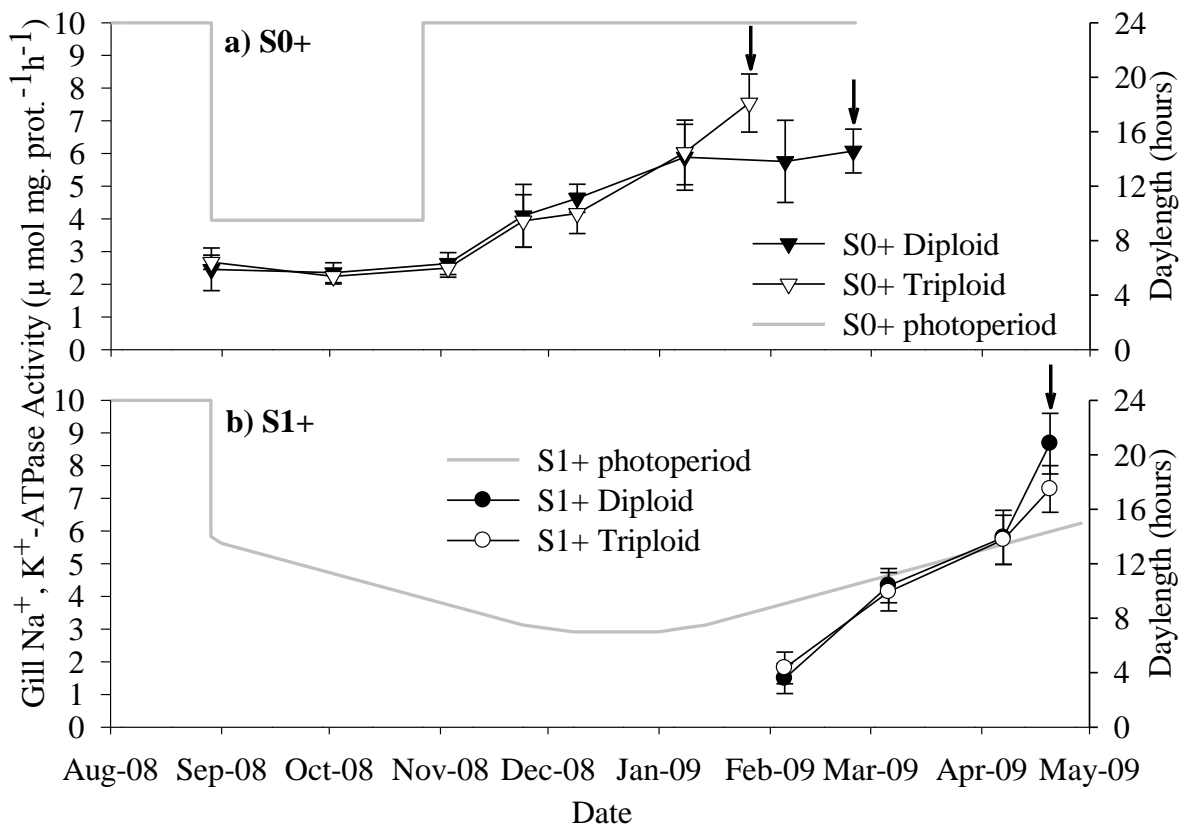
Table 2 summarises the relative attributes of each ploidy-smolt regime at the time of seawater transfer. Weight at smoltification did not differ between smolt regimes within a given ploidy, although triploid S1+ smolts were of comparable size to diploid S0+. S0+ smolts also exhibited higher SGRs than their S1+ siblings but did not differ significantly between ploidies, although there was a trend towards triploids having a higher SGR. Furthermore, S0+ smolts had a significantly higher K than their S1+siblings at sea transfer. Finally, freshwater rearing mortality was very low and did not differ between ploidies or smolt regime (Table 2). Deformity prevalence in both ploidy-smolt groups was also low during the freshwater phase ( $\leq 5\%$ ). Two deformity types were observed, spinal deformity (S1+ 2N=0.4%, 3N=0.8%; S0+ 2N=0.0%, 3N=0.2%) and operculum shortening (S1+ 2N=4.6%, 3N=2.4%; S0+ 2N=1.1%, 3N=1.1%).

**Table 2.** Summary of fish performance (smolt group and ploidy) in fresh and sea water. Superscripts denote significant differences ( $p < 0.05$ ).

	S1+		S0+	
	2n	3n	2n	3n
<b>FRESHWATER (at seawater transfer)</b>				
Weight (g)	35.1 $\pm$ 1.5 <sup>a</sup>	42.2 $\pm$ 1.5 <sup>bc</sup>	39.1 $\pm$ 1.2 <sup>ab</sup>	43.7 $\pm$ 1.2 <sup>c</sup>
SGR (% day <sup>-1</sup> )	0.64 $\pm$ 0.08 <sup>a</sup>	0.62 $\pm$ 0.05 <sup>a</sup>	0.89 $\pm$ 0.09 <sup>b</sup>	0.95 $\pm$ 0.09 <sup>b</sup>
K	1.01 $\pm$ 0.01 <sup>a</sup>	0.96 $\pm$ 0.01 <sup>b</sup>	1.13 $\pm$ 0.01 <sup>c</sup>	1.09 $\pm$ 0.01 <sup>d</sup>
ATPase	8.7 $\pm$ 0.9 <sup>a</sup>	7.3 $\pm$ 0.7 <sup>ab</sup>	6.2 $\pm$ 0.7 <sup>b</sup>	7.5 $\pm$ 0.9 <sup>ab</sup>
Deformities (%)	5.1	3.3	1.3	1.7
Survival (%)	99	99.5	99.5	99
<b>SEAWATER (at end of trial)</b>				
Weight (g)	120.6 $\pm$ 3.3 <sup>a</sup>	135.6 $\pm$ 4.1 <sup>b</sup>	142.5 $\pm$ 3.8 <sup>c</sup>	165.0 $\pm$ 7.1 <sup>d</sup>
SGR (% day <sup>-1</sup> )	1.25 $\pm$ 0.10 <sup>a</sup>	1.26 $\pm$ 0.04 <sup>a</sup>	1.40 $\pm$ 0.07 <sup>b</sup>	1.40 $\pm$ 0.03 <sup>b</sup>
Deformities	1.7	2.5	0.8	2.5
Survival (%)	100	100	99.6	100

### 3.2. Smoltification

S0+ gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was affected by time but not ploidy or family. Activity remained at basal levels from the onset of the short-day photoperiod and increased following the return to LL in late October in both ploidy groups (Fig.3a). S0+ triploid activity increased significantly from October to mid-January ( $p < 0.05$ ) prior to seawater transfer as smoltification was completed, also coincident with a final smolt index score of 4 (data not shown). S0+ diploids showed a slower increase in activity during this period, reaching a significantly higher activity than basal a further 4 weeks later. During this period smolt index for the S0+ diploids was lower ( $\text{SI}=2.5$ ) than triploids but attained a score of 4 at seawater transfer on 19<sup>th</sup> February 2009.



**Figure 3.** Changes in gill ATPase activity (mean  $\pm$  SEM,  $n=8-10$ ) in S0+ (a) and S1+ (b) smolt populations from both ploidies. Arrows indicate SW transfer. No significant differences were found between ploidies.

Similarly gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was significantly affected by time but did not differ between ploidies or families within the S1+ population (Fig.3b). Both ploidies

showed a significant elevation in activity under the increasing natural daylength of spring (early February to late April). Prior to seawater transfer on 22<sup>nd</sup> April all fish had a smolt index score of 4 following a continual development of colouration from January.

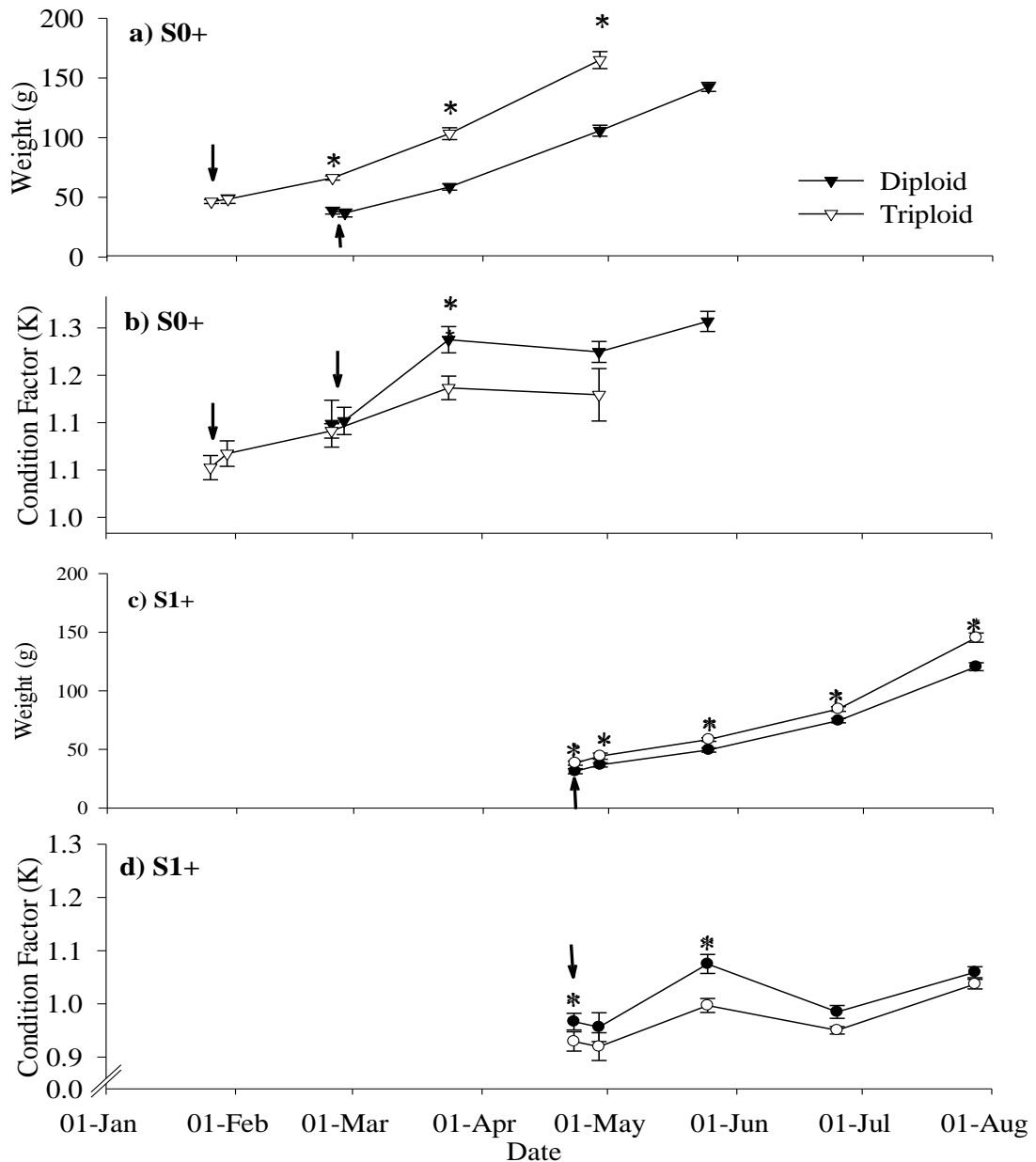
Final gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity on time of seawater transfer did not differ between ploidies or smolt regime, except for S0+ diploids showing a significantly lower activity than their S1+ siblings (Table 2).

No mortalities were recorded following the 96 h seawater challenge in either smolt-ploidy population. Serum osmolality rose from 335±4 mOsm kg<sup>-1</sup> in freshwater to 358±3 mOsm kg<sup>-1</sup> (p<0.05) 48 hours post-seawater transfer in both smolt populations but did not differ significantly between ploidies (data not shown). Seven days post transfer, serum osmolality returned to 331±3 mOsm kg<sup>-1</sup> in both smolt-ploidy populations.

### 3.3. Seawater Growth

During the first 90 days of seawater rearing both triploid S0+ and S1+ post-smolts maintained a significant weight advantage over their diploid siblings (Fig.4a and 4c). However, SGR did not differ between ploidies within a given smolt regime (Table 2), but S0+ post-smolts had a significantly higher SGR than S1+ post smolts. In both smolt regime-ploidy groups, K increased with duration in seawater (Fig.4b and 4d). After 90 days K was significantly higher than at initial transfer in diploid S0+ but not triploid S0+, although there was no significant difference between the two ploidy. In the S1+ population, although fluctuating, K was significantly higher after 90 days than at transfer in both ploidies with no significant difference between the two. Only 1 mortality (diploid S0+) was recorded following seawater transfer and during the

subsequent 3 months of rearing (Table 2). Similarly, deformity during the first 3 months of seawater rearing was low ( $\leq 2.5\%$ ) in either ploidy or smolt population. Again spinal deformity (**S1+** 2N=0.0%, 3N=1.3%; **S0+** 2N=0.5%, 3N=1.3%) and operculum shortening (**S1+** 2N=1.7%, 3N=1.3%; **S0+** 2N=0.5%, 3N=1.3%) were the two main types observed.



**Figure 4.** Changes in growth performance and condition factor (mean  $\pm$  SEM,  $n=30$ /ploidy/family) during the three month following sea transfer in S0+ (a and b) and S1+ (c and d) post-smolt for both ploidy. Arrows indicate SW transfer. Asterisk indicates significant ploidy differences ( $p < 0.05$ ).

#### 4. Discussion

To the authors knowledge we have demonstrated for the first time that triploid Atlantic salmon smolts can be produced out-of-season following a square-wave photoperiod routinely used in commercial diploid stocks. This is of particular significance to the aquaculture industry as it maintains the ability to provide year round production of salmon if they were to adopt the change in ploidy for production. Furthermore, we demonstrated that in both S1+ and S0+ populations that triploids significantly out-grew their diploids siblings during the freshwater phase to seawater transfer (SWT), with the significant weight advantage maintained throughout early seawater rearing and minimal mortality and deformity which is in accordance with a recent study (Oppedal *et al.*, 2003).

As is well established in the literature, accelerated S0+ smolts irrespective of ploidy have a higher SGR than their siblings under a natural S1+ regime (Thrush *et al.*, 1994; Duncan and Bromage, 1998). However, more importantly in our study was that triploid S0+ smolts completed smoltification 4 weeks earlier than their diploid siblings and at a significantly larger size. A strong correlation between size at SWT and survival has been reported (Duston and Saunders, 1990; Handeland and Stefansson, 2001) and may explain why we also observed minimal mortality on SWT unlike previous studies (Galbreath and Thorgaard, 1995; McCarthy *et al.*, 1996; O'Flynn *et al.*, 1997; Benfey, 2001). Furthermore, this differential time of smoltification within ploidy also suggests there may be a different minimum size-specific threshold for smoltification in triploids as known for normal diploid smolt production (Johnson *et al.*, 1986; Withler *et al.*, 1995; Handeland and Stefansson, 2001). S1+ triploids smolted at the same time as their diploid siblings although their growth rate was greatly reduced in comparison with the S0+ population. This suggests that under accelerated regimes, reaching a size specific

threshold may indeed be more important when attempting to produce out-of-season triploids as observed in coho salmon (Johnson *et al.*, 1986; Withler *et al.*, 1995) and suggested in Atlantic salmon (Galbreath and Thorgaard, 1995). Failure to reach such thresholds and incomplete smoltification may explain the commonly reported poor seawater performance observed in many earlier studies. In this respect it will be essential in future studies to define such size thresholds in relation to the onset of out-of-season smolt photo-thermal regimes (Solbakken *et al.*, 1994; Handeland *et al.*, 1998; Handeland *et al.*, 2004; Nordgarden *et al.*, 2007). Furthermore it will be necessary to explore these interactions with lipid status, protein inclusion, feeding regime and ration which are known to have a strong influence on successful smoltification (Nordgarden *et al.*, 2002; Bendiksen *et al.*, 2003; Berrill *et al.*, 2004; Berrill *et al.*, 2006).

On a cautionary note, care must be taken when referring to out-of-season smolts in this study, as though advanced in comparison to their S1+ siblings, they were not true 6-month advanced S0+ smolts as traditionally referred to, as our fish were reared under ambient water temperatures rather than constant warm water (Solbakken *et al.*, 1994; Duncan *et al.*, 1998). It will be imperative that triploid performance be monitored under such accelerated photo-thermal regimes particularly since triploid salmonids have been reported to be more sensitive to environmental changes, especially high temperature, than diploid fish, which may relate to their altered physiology and cellular morphology (Benfey and Biron, 2000; Hyndman *et al.*, 2003b; Atkins and Benfey, 2008). However, the significant finding is that S0+ triploid smolts responded well to an out-of-season photoperiod regime.

One final aspect with regards to growth is that in this particular study we cannot draw firm conclusions as to specific family effects on triploid smolt performance as only two were used, although such effects have already been reported in other studies

(Friars, *et al.*, 2001; Oppedal *et al.*, 2003; Johnson *et al.*, 2004). It will also be essential to consider ploidy-strain interactions as differences in diploid growth performance have been observed between different Atlantic salmon strains (Handeland *et al.*, 2003a; Handeland *et al.*, 2003b; Handeland *et al.*, 2004) as they have in other salmonids such as rainbow trout (*Onchorhynchus mykiss*) (Valente *et al.*, 1999; Henryon *et al.*, 2002; Taylor *et al.*, 2006). Finally, it will also be important to understand muscle fibre recruitment patterns and hypertrophy in triploid salmon as the greater triploid growth we observed may result from increases in such parameters and/or increased feed intake (Johnston *et al.*, 1999) in addition to previously mentioned genetic influences.

Smoltification is normally associated with improved hypo-osmoregulatory ability and decreasing condition factor (Solbakken *et al.*, 1994; McCormick *et al.*, 1995; Duncan and Bromage, 1998). In both ploidy and photoperiod groups, condition decreased during the parr–smolt transformation and a high incidence of smoltification was observed. This was also accompanied by an increase in gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity on the return to LL in the S0+ group or the increase of daylength in spring in the S1+ group in accordance with previous studies (Thrush *et al.*, 1994; Berge *et al.*, 1995; Duston and Saunders, 1995). However, two observations are noteworthy from this study. Firstly, condition did not decrease as much at the time of smoltification in S0+ smolts compared to their S1+ siblings, and secondly, gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity was generally lower in S0+ smolts.

Given that condition factor correlates with lipid content (Herbinger and Friars, 1991), it may be that accelerated production regimes do not allow individuals to mobilise long-term lipid reserves to aid the development of hypo-osmoregulatory mechanisms, favouring short-term stores such as the liver (Storebakken and Austreng, 1987; Berrill *et al.*, 2006). Similarly Nordgarden *et al.* (2002) did not observe a change

in the levels of muscle or body lipid during smoltification in 0+ Atlantic salmon smolts, while for smolts produced under a natural S1+ regime, reductions in muscle, liver and visceral fat are well documented (Helland and Grisdale-Helland, 1998). As such it may not be appropriate to use condition as an accurate measure of smoltification (Berrill *et al.*, 2006). Certainly future studies examining this aspect with regards to lipid mobilisation are warranted.

Secondly, the magnitude of the increase and final pre-transfer levels of gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity were lower than generally reported, although there is a large variability within the literature (McCormick, 1993; McCormick *et al.*, 2000; Handeland and Stefansson, 2001). This is significant as it shows the higher ATPase activity (>10  $\mu$  mol mg. prot.<sup>-1</sup>h<sup>-1</sup>) considered within the industry as indicative of 'smolts' may not apply to triploid smolts, or smolts in general. However, this may be a function of size as Handeland and Stefansson (2001) found lower gill activity in large rather than small smolts, and proposed smaller smolts have a greater area/volume ratio. In accordance, our results showed that smaller S1+ smolts generally had a higher gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity than the larger S0+ smolts, certainly in the case of diploids. This could indicate that smaller overall gill surface area of S1+ smolts requires, despite their greater gill area/volume ratio, increased activity to bring about correct osmoregulatory function. Further research is required to assess the relationship between gill surface area, body weight and osmoregulatory capacity, including chloride cell size and density and ATPase activity. In addition, smoltification in the S0+ smolts occurred after 450°DD and 510°DD following the return to LL after the short-day period for triploids and diploids respectively, which is later than commonly reported where smoltification is completed within 400°DD (Handeland and Stefansson 2001). Since ambient water temperatures were below 4°C from mid-December onwards (mid-way through LL



period) this may explain the delay in the parr-smolt transformation, as low temperatures are known to slow the process (McCormick *et al.*, 2000). Similarly, an early increase in temperature from 5 to 12 °C advanced development of seawater tolerance in Atlantic salmon smolts compared with controls raised at ambient water temperature (5–6 °C) (Solbakken *et al.*, 1994). Furthermore, as S0+ diploids were smaller than their triploid siblings during this period, and that larger fish develop hypo-osmoregulatory capacity earlier than smaller ones (Handeland and Stefansson 2001), this concurs with the idea of why the diploids smolted later. This also again lends support to the idea that triploids may have size-specific thresholds different to those of diploids. Overall, the SWT mortalities described by Galbreath and Thorgaard (1995), McCarthy *et al.* (1996) and Benfey (2001), were not observed in the present experiment and indicates that we produced fully adapted saltwater tolerant triploid smolts.

Previous studies have linked triploid induction with increases in the prevalence of skeletal and anatomical deformities within the salmon aquaculture industry. Such deformities may impact feeding opportunity, swimming and respiratory abilities, and ultimately compromise survival. In addition to ethical concerns for animal welfare, such deformities reduce the aesthetics and the commercial value of the fish (Ornsrud *et al.*, 2004). Lower jaw deformity (LJD) has been the most frequently reported skeletal abnormality in triploid Atlantic salmon (Sutterlin *et al.*, 1987; Jungalwalla, 1991; McGeachy *et al.*, 1996; Lijalad and Powell, 2009), although cataract, reduced gill surface area (GSA), gill filament deformity syndrome (GFD) and various other spinal deformities are also readily reported (Sadler *et al.*, 2001; Oppedal *et al.*, 2003). However, of significant interest was that in the present study the prevalence of deformity in both fresh and seawater rearing was very low ( $\leq 5\%$ ) and did not differ by ploidy, which is comparable to the most recent study by Oppedal *et al.* (2003).

Furthermore, the most common morphological anomaly apparent within our study was opercula shortening, which may be in fact an artefact of feeding-induced collision-related damage, or excessive flow rates during early rearing rather than a “true” deformity (Abbot *et al.*, 1994; Tave and Handwerker, 1994; Turnbull *et al.*, 1998). However, vitamin and mineral deficiency in the diet can also induce deformation of the opercula (Dabrowski *et al.*, 1988). In addition, temperature during egg incubation and early life-stages, both in term of absolute value and variations, was also shown to induce bone and opercula deformities (Ørnsrud *et al.*, 2004; Wargelius *et al.*, 2004). Although actual rate of deformities were low throughout the trial, the higher proportion of deformity observed in triploids suggests their higher susceptibility to temperature-induced deformity. Clearly further studies are required to elucidate the cause of such malformations before triploidy can be adopted as a successful strategy, although the fact that ploidy effects were not apparent in this study is highly encouraging.

Overall, our results are promising for the salmon industry as they indicate year round production of salmon can be maintained and genetic/ecological threats reduced through the potential adoption of triploid salmon. It must be said that not only would genetic and ecological threats to wild populations be minimised, triploidy could also offer several other benefits such as increased somatic growth due to extended grow-out as diversion of energy into gonadal tissue is avoided, wider harvest windows, reduced running costs as the use of photoperiod regimes at sea to suppress early maturation would potentially no longer be required and last but not least, it would offer a means to salmon breeding companies to protect their IPR on selected strains. There is however still a long way to go before triploidy can become a commercial reality within the salmon industry. Further studies are essential to establish optimized breeding programs,

refine rearing requirements as well as understand the cellular and molecular regulation of physiological function.

### **Acknowledgements**

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## CHAPTER 6

### PAPER VIII

#### *RESEARCH ARTICLE*

#### COMPARATIVE SEAWATER PERFORMANCE AND DEFORMITY PREVALENCE IN OUT-OF-SEASON DIPLOID AND TRIPLOID ATLANTIC SALMON (*SALMO SALAR*) POST-SMOLTS.

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**Contributions:** The present manuscript was compiled and written in full by the author of this thesis with the help from supervisors. The candidate of the present thesis performed the triploidization and sampling, analyzed heart, jaw and gill morphometry, performed statistical analyses with the help and guidance from the supervisors (Drs. Herve Migaud and John Taylor). X-radiography analyses were performed in Norway (Institute of Marine Research, IMR) by UoS MS.c. student Damian Fison under the supervision of Dr. Herve Migaud (at UoS) and Ton Hansen and Per Gunnar Fjellidal at IMR. Meritxell Diez-Padrisa performed the eye histology and diagnostic.

**Keywords:** Atlantic salmon, triploid, growth, deformity, vertebrae, cataract, family.

**Abstract**

The use of sterile triploid stock in the Atlantic salmon (*Salmo salar*, L) farming industry is the only commercially available means to prevent the ecological impact of domesticated escapees. This study compared the seawater (SW) performance and deformity prevalence of diploid and triploid post-smolts from 2 full-sib families produced out-of-season. Triploids completed smoltification 4 weeks earlier and at a significantly higher body-weight. Growth and survival in SW were not significantly affected by ploidy. The incidence of external deformities, dominated by jaw malformation, was ~12% in triploids and below 5% in diploids. Vertebral deformities were more prevalent in the fastest growing triploid family only. Heart morphometry differed between ploidies which may relate to a higher cardiac workload in triploids. No clear alteration of the gill apparatus was detected. The most significant detrimental effect of triploidy was on the rate and severity of cataract that were observed from August onward (50% and 92% of diploids and triploids respectively affected after 1-year in SW). At that time, cataracts were diagnosed by histological examinations as irreversible with a probable osmotic origin which could arise from factors such as water quality, nutritional deficiencies or thermal variations. This study warrants further research aiming at adapting rearing practices to the needs of triploid stocks as to improve their performance and welfare.

## 1. Introduction

The increase in the global volume of farmed Atlantic salmon, *Salmo salar*, has given rise to increasing public and scientific awareness over the impact of farmed stocks on the integrity of wild populations (Glover *et al.*, 2009). In the North Atlantic, an estimated two-million Atlantic salmon escape annually (McGinnity *et al.*, 2003) and circa 1.9 million escapees were reported in Scotland alone between 2002 and 2008 with the vast majority on the west coast (Marine Scotland, 2010). Due to decades of domestication and selective breeding for economically driven traits (Gjøen and Bentsen, 1997), commercial strains of Atlantic salmon have a reduced genetic variation (Skaala *et al.*, 2005), but are capable of survival, dispersion, homing and successful spawning upon escape (Lura and Sægrov, 1991; Webb *et al.*, 1991, 1993; Hansen *et al.*, 1993; Hansen, 1996; Hansen and Youngson, 2010). Interbreeding between wild and farmed strains could therefore reduce the fitness of the wild stock through genetic introgression (Crozier 1993; McGinnity *et al.*, 2003; Skaala *et al.*, 2006; Castillo *et al.*, 2008; Roberge *et al.*, 2008). By providing genetic and reproductive containment, sterilization is the only reliable means to prevent such interbreeding and the propagation of non-native salmon (Fleming *et al.*, 1996; Piferrer *et al.*, 2009).

Triploidization of newly fertilised eggs by hydrostatic pressure shock is an effective and well established protocol in Atlantic salmon to induce sterility (Benfey and Sutterlin, 1984; McGeachy *et al.*, 1995; Benfey 2001; Piferrer *et al.*, 2009). For the on-grower, a sterile population could eliminate sexual maturation with its negative impact on growth, health, welfare and overall value of the stock (Kadri *et al.*, 1996; St-Hilaire *et al.*, 1998; Leclercq *et al.*, 2010; Taranger *et al.*, 2010). Despite the potential benefits, the sterilization of Atlantic salmon is seldom applied commercially unlike in other salmonids such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo*

*trutta*) and brook trout (*Salvelinus fontinalis*) (Piferrer *et al.*, 2009). Only the Australian industry has adopted this strategy (Sadler *et al.*, 2000; Sadler *et al.*, 2001; Lijalad and Powell, 2009). The main explanation for the lack of commercial implementation is the reduced culture performance of triploid stocks observed in previous studies and commercial trialing over the last two decades as well as consumer perception (Benfey, 2001; Oppedal *et al.*, 2003). The reduced performance documented in triploids could result from their larger cell volume and reduced cell number suggesting a reduced cell surface area to volume ratio and a lower capacity for cellular metabolic exchange (Suresh and Sheehan, 1998; Hyndman *et al.*, 2003; Benfey and Bennett, 2009). This could also affect the physiology and especially organ development and morphometry of triploids but evidences remain scarce to date. Variations in muscle fiber population and morphometry were documented in triploid salmonids (Suresh and Sheehan, 1998; Johnston *et al.*, 1999). The density of satellite cells was shown to be approximately 24 % lower in triploid than in diploid individuals, and triploids recruited one-third fewer fibers for each mm<sup>2</sup> increase in white muscle cross-sectional area (Johnston *et al.*, 1999). A faster growth of triploids by muscle hypertrophy (volumetric growth) was described and shown to positively affect flesh quality (Poontawee *et al.*, 2007). Overall, triploids have been reported to show higher (O'Flynn *et al.*, 1997; Oppedal *et al.*, 2003) or lower (Whitler *et al.*, 1995; Cotter *et al.*, 2002; Shrimpton *et al.*, 2007) growth. They have also been shown to have a similar or lower survival than their diploid siblings with events of high triploid mortality at sea being documented (Galbreath *et al.*, 1994; Galbreath and Thorgaard, 1995; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997). Survival and growth decreased more rapidly in triploids than diploids subjected to sub-optimal conditions (Ojolick *et al.*, 1995) such as high water temperature (Altimiras *et al.*, 2002; Atkins and Benfey, 2008). However, it is the prevalence of deformity that has

significantly hindered the adoption of triploidy by the industry (Taranger *et al.*, 2010). Reported deformities include jaw deformity, opercular shortening, gill filament deformity syndrome and reduced gill surface area, vertebral deformities (Sadler *et al.*, 2001; Lijalad and Powell, 2009; Taranger *et al.*, 2010) and ocular cataracts (Wall and Richard, 1992; Oppedal *et al.*, 2003). Vertebral deformities include macroscopic curvature of the vertebral column such as scoliosis and lordosis (O'Flynn *et al.*, 1997) and a range of deformational changes only identifiable by radiological examination (Witten *et al.*, 2009). These can have implications on the swimming performance and present a metabolic cost (Lijalad and Powell, 2009; Powell *et al.*, 2009) but can also affect the market value and arguably the animal welfare.

Importantly, some studies have shown no effect of triploidization on mortality and deformity prevalence (Oppedal *et al.*, 2003) while, conversely, similar levels of deformity have been observed in diploids (Sutterlin *et al.*, 1987; Kacem *et al.*, 2004). It was suggested that deformities, even when occurring in triploid siblings only, might not be the result of the triploid induction method *per se* (Taranger *et al.*, 2010). Triploids should be treated as a ‘‘new species’’ (Benfey, 2001) due to their specific environmental, nutritional and physiological needs. Furthermore, a strong effect of the parental origin on the performance of triploid salmonids was characterized in various studies leading to the recognized need to establishing breeding programs specifically designed to improve triploid performance (Friars *et al.*, 2001; Oppedal *et al.*, 2003; Johnson *et al.*, 2004). Furthermore, previous studies reporting lower survival and growth of triploids compared to diploids have sometime used a communal rearing design (Ojolick *et al.*, 1995; Withler *et al.*, 1995). This might have impaired transfer to seawater (SW) of each ploidy at the optimal time and caused detrimental interactions between ploidies. It was indeed shown that triploid salmonids were more likely to be the



recipient of agonistic behaviour (Carter *et al.*, 1994) and were less aggressive during feeding (Garner *et al.*, 2008).

Sterilization through triploidization is increasingly regarded as a necessary step to isolate domesticated Atlantic salmon stocks, prevent their ecological impacts and increase the sustainability of the growing Atlantic salmon industry. Using Scottish stocks supplied from an established diploid selective breeding program, this study aimed at assessing the effects of family and ploidy on survival, growth performance and deformity prevalence in out-of-season post-smolts.

## **2. Materials and Methods**

### **2.1. Fish stock and husbandry**

On 5<sup>th</sup> December 2007, two female and two male 2 sea-winter Atlantic salmon broodstock were selected from an established Scottish selective breeding program (Landcatch Ltd., Ormsary, UK) and stripped. Eggs (~3000) and milt (~ 4 ml) from each parent were divided volumetrically into 2 sub-batches and two full-sib families were produced (1 male: 1 female; F1 and F2) by dry fertilisation. Triploidy was induced in one sub-batch per family at 300 °C min post-fertilisation (30 min at 10 °C) by applying a hydrostatic pressure shock (5 min at 65,500 kPa; Johnstone and Stet, 1995) using a customized pressure chamber. Fertilised ova were then water hardened and each family/ploidy group was stocked in isolation into separate hatchery troughs (Howietoun Hatchery Unit, Sauchie, UK) for incubation under controlled temperature (at  $8 \pm 0.5$  °C) until hatch and first feeding (14<sup>th</sup> April 2008). Triploidy success rate was determined at the FW stage by measuring the length of the red blood cell (RBC) nucleus on Giemsa stained blood smears (Johnsson *et al.*, 2004). Pressure shocked groups consistently showed a longer RBC nuclear length (mean value: triploid:  $9.64 \pm 0.54$  µm, diploid:

$7.36 \pm 0.48 \mu\text{m}$ ;  $p < 0.001$ ,  $n = 60/\text{ploidy}$ ) such that the triploidy success rate was 100% in all samples analysed. Parr were transferred to the freshwater (FW) Niall Bromage Freshwater Research Facility (NBFRF; Buckieburn, UK) on 15<sup>th</sup> May 2008 where they were maintained on continuous light (LL) prior to an out-of-season (S0) photoperiod smolt regime.

On 29<sup>th</sup> August 2008, three batches of 150 fish were randomly selected from each family-ploidy group and placed into triplicate 0.9 m<sup>3</sup> tanks ( $n = 3$  tanks/family/ploidy, total of 12 tanks) with a flow of 10 L/min. Fish were fed Nutra Parr starter (Skretting, Invergordon, UK) over the normal daylight period according to the manufacturer's recommendations with an ambient temperatures ranging from 16 °C (summer peak) to 2 °C (at time of SW-transfer). To produce out-of-season (S0+) smolts, fish were maintained on artificial short-days (LD 9.5: 14.5) for a period of eight weeks from 29<sup>th</sup> August until 24<sup>th</sup> October, before being returned to LL until smoltification was completed. Smoltification was determined by assessing gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (McCormick, 1993) and skin silvering (Sigholt *et al.*, 1995) at regular intervals prior to SW-transfer (SWT).

## 2.2. Experimental Design

The stock was transferred to SW (Machrihanish Marine Environmental Research Laboratory, MERL, Scotland) on 22<sup>nd</sup> January and 19<sup>th</sup> February 2009 for triploids and diploids respectively. Fish were stocked into four 2 m<sup>3</sup> SW tank keeping ploidies but not families discrete ( $n = 2$  tanks/family/ploidy, 120 fish/family/tank) with a flow rate of 20 L/min. Differentiation between families was achieved by removal of the adipose fin of family 2. Due to increasing densities, fish were transferred in June 2008 into 6 m<sup>3</sup> tanks (maintaining the previously described experimental groups) and on-grown until

December 2009 when the experiment was terminated. Post-smolts in SW were reared under natural-photoperiod (NL) and fed a 1:1 mixture of Ecolife HR (Biomar, Grangemouth, UK) and Atlantic smolt (Skretting, Invergordon, UK) using clockwork belt feeders according to the manufacturer's recommendations with ambient temperatures ranging from 6 to 16 °C.

### **2.3. Sampling protocol**

Sampling was performed at monthly intervals in FW ( $n = 3$ ; 20/family/ploidy/replicate). Sampling in SW was performed at two and seven days post-SWT, at monthly intervals for the first 6 months and every two months for the remainder of the trial ( $n = 2$ ; 30/family/ploidy/replicate). At each sampling point, randomly selected fish were anaesthetized (1:10,000 of 2-phenoxyethanol; Sigma-Aldrich, Poole, UK) and measured for whole body-weight (BW;  $\pm 0.1$  g) and fork-length (FL;  $\pm 0.1$  cm). The occurrence of external deformities and ocular cataract was assessed visually as described thereafter. Fish were then recovered in clean aerated water and returned to their original tank.

At the end of the trial in December 2009, fish were killed ( $n = 2$ ; 55/family/ploidy/replicate) with an anaesthetic overdose and assessed for body-size, external deformity and macroscopic cataract as described thereafter. On randomly selected sub-samples, digital pictures of the left-hand side anterior body-portion were taken (20/family/ploidy/replicate) for later image analysis of the jaw morphology. The whole heart and the whole left hand-side gill apparatus (10/family/replicate/ploidy) were preserved in 10% buffered formalin until analysis. Selected eyes (3 to 5/macroscopic cataract score/ploidy) were enucleated and preserved in Bouin's fixative for later histological analysis aiming at describing occurring cataracts against their

macroscopic scoring. Finally, whole carcasses (10/family/ploidy/replicate) were frozen at -20 °C for later radiological analysis of vertebral deformities.

## 2.4. Parameters analysed

### Survival and growth

Survival from egg fertilisation to transfer to NBF<sub>RF</sub> was circa 15% with no effect of family and ploidy (data not shown). Survival was then assessed from the parr-stage to smoltication (FW survival) and from SWT to trial completion (SW survival). Fulton's condition factor (K) was calculated as  $K = (100 \text{ BW}) / \text{FL}^3$ ; where BW is whole body-weight (g) and FL is fork-length (mm). Specific growth rate (SGR) over the FW and SW experimental period was calculated as  $\text{SGR} = 100 (e^g - 1)$ ; where  $g = (\ln \text{BW}_f - \ln \text{BW}_i) / (t_f - t_i)$ ,  $\text{BW}_f$  and  $\text{BW}_i$  are the mean final and initial BW respectively and  $(t_f - t_i)$  is the duration of the growth period in days. The mean growth rate in SW was also measured as the thermal growth coefficient (TGC) to account for the earlier SWT of triploids as follow:  $\text{TGC} = (\text{BW}_f^{1/3} - \text{BW}_i^{1/3}) * (1000/\text{DD})$ ; where  $\text{BW}_f$  and  $\text{BW}_i$  are as previously addressed for SGR and DD is the cumulative daily water temperature (°C) in SW.

### External deformity and jaw morphometry

The occurrence and type of external deformities were assessed visually and included spinal deformities (trunk or tail area; curvature or shortening), jaw deformities (lower or upper, shortening or twisting) and opercular shortening (single or both opercula). Digital pictures of the anterior body-portion were analysed by image analysis (Image ProPlus 4.5; Mediacybernetics, MD, USA) according to Lijalad and Powell (2009). The distances between the base of the pectoral fin and the tips of the lower (L2)

and upper (L1) jaws were measured to calculate the lower jaw index (LJI) as  $LJI = L2 / L1$ ; where lengths are expressed in pixels. A threshold value of 0.94 below which a fish was considered to have a jaw deformity was used (H. King, pers. comm., Tasmania).

### **Heart and gill morphometry**

The heart was excised and gently squeezed to expel the blood. The whole heart wet weight (Atrium, bulbus arteriosus and ventricule) was determined ( $\pm 0.01\text{g}$ ) to calculate the cardio-somatic index (CSI) as  $CSI = (100 HW) / BW$ ; where HW is the whole heart wet-weight (g) and BW is the whole body-weight (g) (Bell *et al.*, 1992; Powel *et al.*, 2002). Each heart was then digitally imaged with the cranio-ventral surface uppermost then in lateral recumbency in order to measure the ventricule height:width ratio and the alignment (angle) of the bulbus arteriosus respectively by image analysis (Image ProPlus 4.5) according to Poppe *et al.* (2003).

The second gill arch (from the outer body surface of the apparatus) was dissected from the whole left hand-side gill apparatus and laid flat on a copy stand to expose the maximum two-dimensional area of the branchial arch. A digital picture incorporating a calibration scale was taken using a standard focal length. The total two-dimensional arch surface area (modified from Sadler *et al.*, 2001), the length of the gill arch bone, the total number of filaments and the length of every 5 filaments was measured by image analysis (Image ProPlus 4.5; modified from Stevens and Sutterlin, 1999). The filament number 0 was defined as located on the tip of the arch bone curvature from which positive numbers were given toward the ventral-end and negative numbers toward the dorsal-end of the apparatus. At any given location, only the longest filament from both hemibranches was measured.

### **Macroscopic and microscopic cataract scorings**

Visual inspection for macroscopic cataract was performed on both eyes using a hand-held ophthalmoscope. The severity of the cataract was scored for each eye according to a previously published scoring index (Wall and Bjerkås, 1999; Macroscopic cataract score 0 = 0%, 1 = 1 to 10%; 2 = 10 to 50%; 3 = 50 to 75%, 4 = 75 to 100% of the lens covered by cataract) then averaged for each fish.

Selected eyes were preserved in Bouin's fixative for 36h then rinsed and stored in 70% ethanol until histological processing. Individual eyes were embedded in wax blocks for sectioning along the visual axis at 5 µm thickness. Sections were stained with Mayer's haematoxylin and eosin Y and examined by light microscopy (Wall and Richards, 1992). The type, position and severity of the observed cataractous changes were determined according to Wall and Richards (1992) but with a maximum severity extended from 3 to 4 in order to match the amplitude of the macroscopic scale (microscopic cataract score 0: absent, 1: slight, 2: moderate, 3: severe, 4: intense cataract).

### **Vertebral deformity**

Frozen carcasses were defrosted and carefully filleted to obtain a clear radiography of the vertebral column using a portable X-ray apparatus (HI-Ray 100, Eickenmeyer Medizintechnik für Tierärzte e.K., Tuttlingen, Germany). The 30×40 cm film (IX 100, FUJIFILM Corp., Tokyo, Japan) was exposed once at 50 mAs and 68 Kv from a height of 90 cm and developed using a manual developer (Cofar Cemat C56D) with Kodak Professional manual fixer and developer. Developed radiographs were digitized by scanning at 300 dpi for examination of vertebral deformities. The total number of vertebrae (V) per fish and the location and type of deformity classified

according to Witten et al. (2009) were recorded. The vertebral column was divided into 4 regions (R) according to Kacem *et al.* (1998): R1 (cranial trunk, V1→V8), R2 (caudal trunk, V9→V30), R3 (tail, V31→V49) and R4 (tail fin, V50→V57/58/59).

## 2.5. Statistical Analysis

A chi-square test of independence was used to determine statistical differences in the relative proportions of observed deformity and cataract between experimental groups. Differences in the mean of the experimental groups were assessed by mixed model analysis of variance (ANOVA) manipulated by a general linear model considering family as a random effect and ploidy as a fixed effect, with replicate tanks nested within family-ploidy grouping. The effect of ploidy and family on gill morphometry was tested by ANCOVA using BW as covariate. Prior to analyses, datasets were checked for normality using the Kolomogorov-Smirnov test and for homogeneity of variance using Levene's tests and observations of residual plots. Log or square-root transformations were applied when required and proportions were arcsine transformed. Post-hoc analyses were carried out using Tukey's multiple comparison tests. Mean cataract score was assessed using a non-parameteric Kruskal Wallis Test with Dunn's Multiple Comparison post-hoc. All statistical comparisons were performed using Minitab v.15 with a significance level of 5% ( $p < 0.05$ ). Results are presented as mean  $\pm$ SEM.

### 3. Results

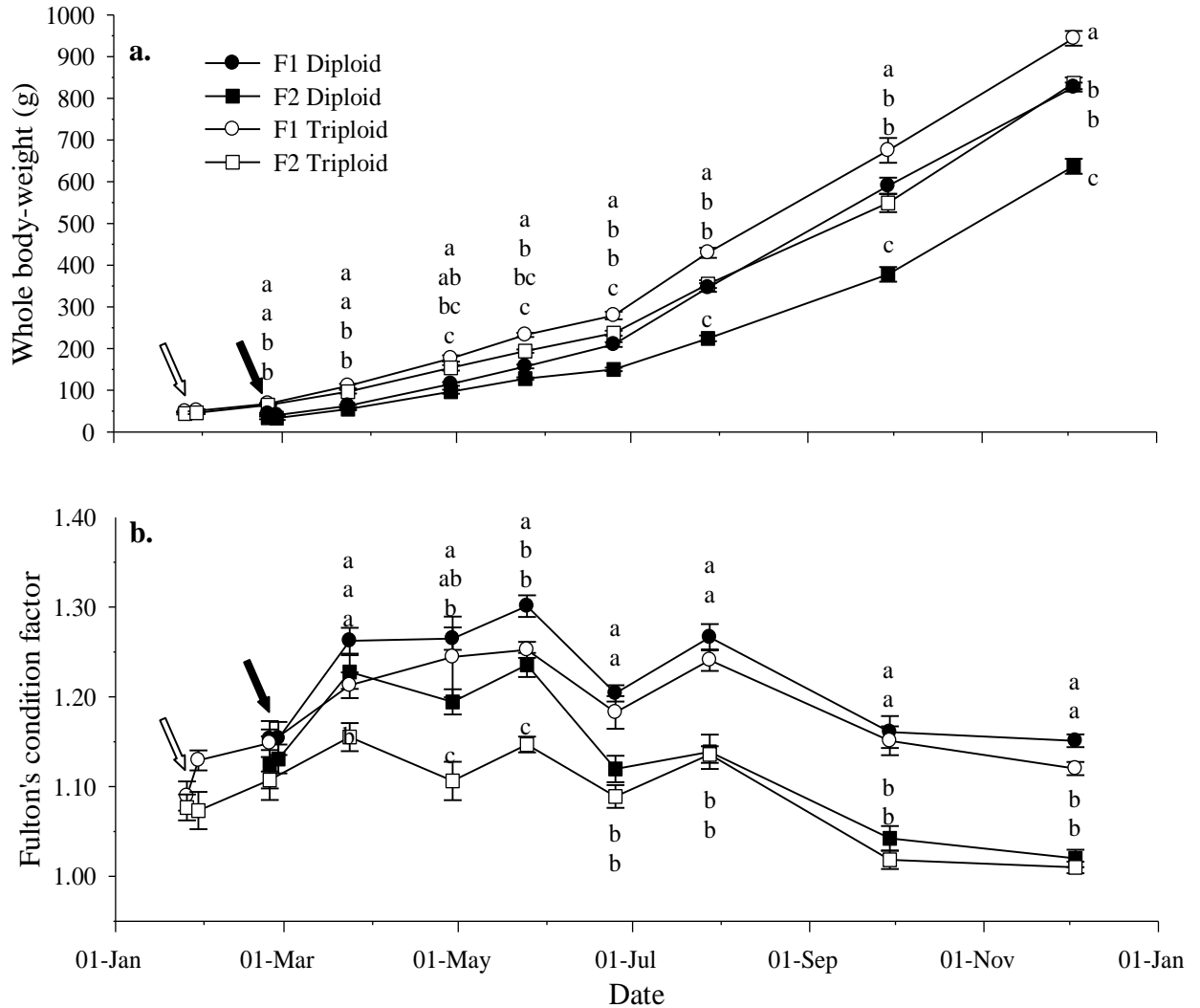
#### 3.1. Survival and growth

Survival ranged between 99% and 99.5% from the parr-stage to SWT and was constant across our experimental groups at 98% during the first year at sea (Table 1a, 1b).

Prior to SWT, triploid smolts had a significantly higher BW than diploids (F1: +22.3%; F2: +29.0%) and, within both ploidies, F1 was always significantly heavier than F2 (Diploid: +15.1%; triploid: +9.1%; Table 1a). Triploids had a significantly lower K than diploids, and within triploids F1 had a significantly higher K than F2. Triploids completed smoltification and were transferred to sea 4 weeks earlier than diploids.

Triploids remained heavier than their sibling diploids throughout the first year in SW (Fig. 1a; Table 1b). At the end of the trial, triploids were significantly heavier than diploids within both families (F1: +14.1%; F2: +31.2%). However triploids were transferred earlier in SW and, over the SW period, SGR was always lower in triploids than diploids (F1: -8.7%, F2: -3.1%) and triploids TGC was lower (F1: -2.5%) or higher (F2: +5.3%) depending on the family. An effect of family on BW was observed within both ploidies from 4-month post-SWT (F1>F2). Accordingly, SGR and TGC were higher in F1 compared to F2 and, at the end of the trial, F1 was significantly heavier than F2 within both ploidies (diploid: +29.8%; triploid: +12.9%; Table 1b). Over most of the SW rearing phase, there was no effect of ploidy on K, but an effect of family within both ploidies was observed at the end of the trial (F1>F2; Fig. 1b, Table 1b).





**Figure 1.** (a.) Whole body-weight (g) and (b.) Fulton's condition factor (K) of the full-sib family-ploidy groups of out-of-season Atlantic salmon post-smolt during their first-year at sea. Values are expressed as mean  $\pm$  SE ( $n = 2, 110$  individuals/family/ploidy). Different superscripts denote significant differences between experimental groups within each sampling point (ANOVA,  $p < 0.05$ ). F: family.

**Table 1:** Growth performance and survival of each family (F)-ploidy group of Atlantic salmon at **a.** the end of their freshwater (FW) life-stage and **b.** the end of their first year in seawater (SW). Values are given as mean  $\pm$  SE or proportions. Note: (a.) the last common sampling point in FW was used to compare whole body-weight (BW) and Fulton's condition factor (K); specific growth-rate (SGR) and survival in FW were determined from the parr-stage to SW-transfer. TGC: Thermal growth coefficient. Different superscripts denote significant differences between experimental groups (ANOVA,  $p < 0.05$ ).

		Diploid		Triploid	
		F1	F2	F1	F2
<b>a. Freshwater performances (n = 60/family/ploidy)</b>					
BW	(g)	37.3 $\pm$ 0.7 <sup>a</sup>	32.4 $\pm$ 0.7 <sup>b</sup>	45.6 $\pm$ 0.7 <sup>c</sup>	41.8 $\pm$ 0.7 <sup>d</sup>
K		1.14 $\pm$ 0.01 <sup>a</sup>	1.12 $\pm$ 0.01 <sup>ab</sup>	1.10 $\pm$ 0.01 <sup>b</sup>	1.08 $\pm$ 0.01 <sup>c</sup>
Survival	(%)	99.5	99.5	99.0	99.0
<b>b. Seawater performances (n = 110/family/ploidy)</b>					
BW	(g)	827 $\pm$ 11 <sup>a</sup>	637 $\pm$ 18 <sup>b</sup>	944 $\pm$ 18 <sup>c</sup>	836 $\pm$ 14 <sup>a</sup>
K	(days)	1.15 $\pm$ 0.01 <sup>a</sup>	1.02 $\pm$ 0.01 <sup>b</sup>	1.12 $\pm$ 0.01 <sup>a</sup>	1.01 $\pm$ 0.01 <sup>b</sup>
SGR	(% day <sup>-1</sup> )	1.04	0.97	0.95	0.94
TGC		2.36	2.08	2.30	2.19
Time in SW	(days)	292	292	320	320
Survival	(%)	98.0	98.0	98.0	98.0

### 3.2. External deformity and jaw morphometry

The prevalence of external deformities (excluding ocular cataracts) at the end of the first year in SW (110 fish assessed/family/ploidy) was significantly higher in triploids (F1: 12.7%; F2: 11.8%) than diploids (F1: 0.9%; F2: 4.6%; Table 2a), with no statistical effect of family within both ploidies. In both triploid families the most common external deformity was a twisting of the lower jaw (14/27 deformities), followed by a shortening of the upper jaw (10/27 deformities). In diploids, it was a shortening of the upper jaw (5/6 deformities). Only one case of gill filament deformity syndrome (GFDS) and one case of observable vertebral deformity (short trunk) were observed (F1 triploids). In comparison, external deformities were significantly lower in both ploidies at the end of the FW stage, with a prevalence of 0.8% in diploids and 2.5% in triploids (data not shown). No fish displayed a lower jaw index (LJI) below the

arbitrary threshold of 0.94. However, LJI was significantly affected by family within both ploidies (F2>F1) and by ploidy within F1 (F1 triploid>F1 diploid; Table 2a).

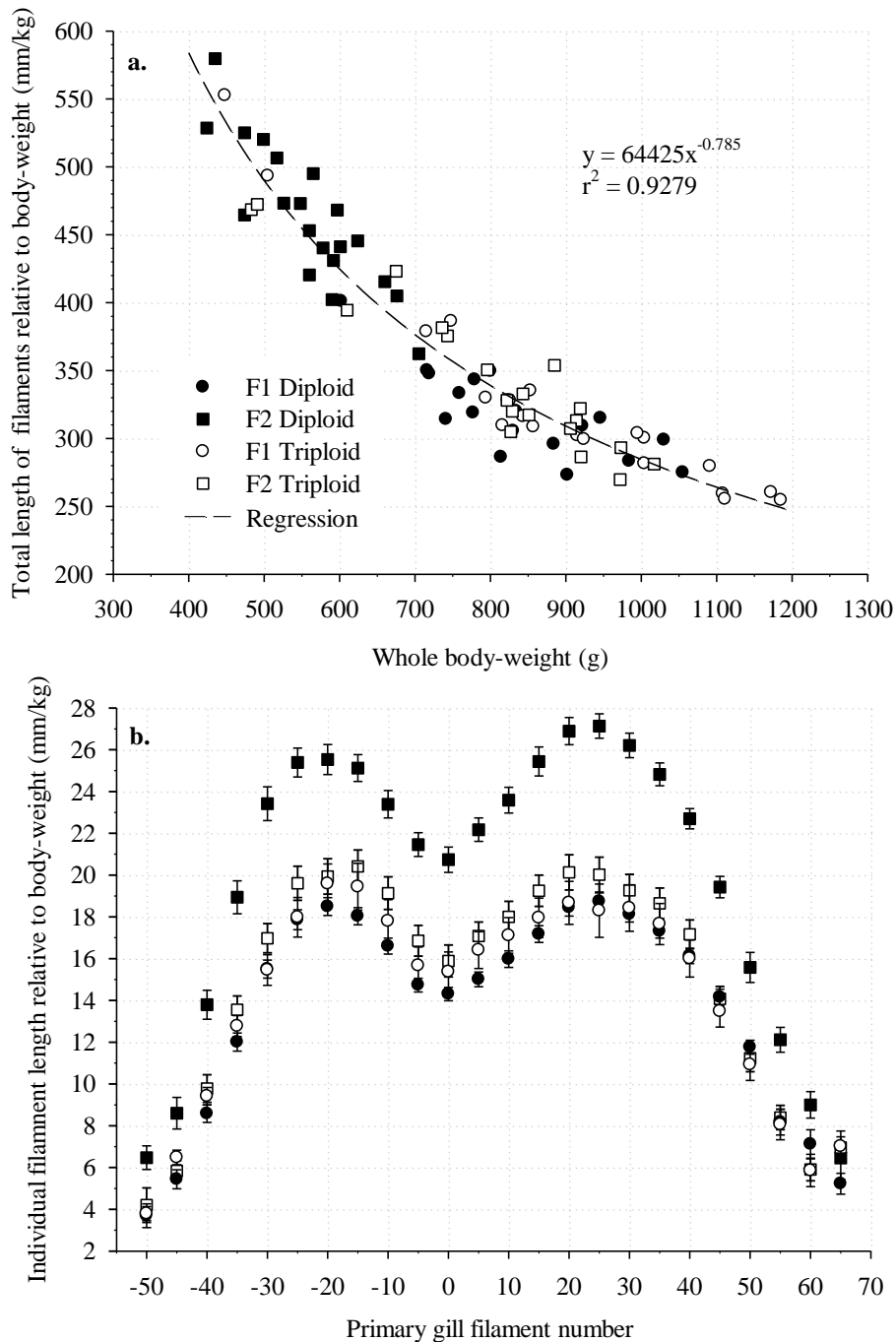
**Table 2.** (a.) Prevalence of external deformities and lower jaw index (Ljalad and Powell; 2009, (b.) heart morphometry (Poppe *et al.*, 2003), (c.) gill morphometry, (d.) prevalence of each macroscopic cataract score (Wall and Bjerkås, 1999) and (e.) number of vertebrae and prevalence of vertebral deformity (observed by radiography) within each family-ploidy group of Atlantic salmon at the end of their first year at sea. Values are given as mean  $\pm$  SE or proportions. Different superscripts denote significant differences between experimental groups (ANOVA for means and Chi-square test for proportions,  $p < 0.05$ ). F: family.

		Diploid		Triploid	
		F1	F2	F1	F2
<b>a. External deformity (n = 110 /family/ploidy) and lower jaw index (n = 40 /family/ploidy)</b>					
External deformity	(%)	0.9 <sup>a</sup>	4.6 <sup>a</sup>	12.7 <sup>b</sup>	11.8 <sup>b</sup>
Lower jaw index		0.985 $\pm$ 0.001 <sup>a</sup>	0.997 $\pm$ 0.002 <sup>bc</sup>	0.994 $\pm$ 0.002 <sup>b</sup>	1.003 $\pm$ 0.002 <sup>c</sup>
<b>b. Heart morphometry (40/family/ploidy)</b>					
Cardio-somatic index		0.15 $\pm$ 0.005 <sup>a</sup>	0.177 $\pm$ 0.006 <sup>b</sup>	0.167 $\pm$ 0.005 <sup>ab</sup>	0.171 $\pm$ 0.004 <sup>ab</sup>
Height	(mm)	14.4 $\pm$ 0.03 <sup>a</sup>	12.7 $\pm$ 0.02 <sup>b</sup>	15.4 $\pm$ 0.03 <sup>a</sup>	14.4 $\pm$ 0.03 <sup>a</sup>
Width	(mm)	14.0 $\pm$ 0.03 <sup>a</sup>	12.7 $\pm$ 0.02 <sup>b</sup>	14.0 $\pm$ 0.03 <sup>a</sup>	12.9 $\pm$ 0.02 <sup>b</sup>
Height/Width		1.04 $\pm$ 0.02 <sup>ab</sup>	1.00 $\pm$ 0.02 <sup>a</sup>	1.10 $\pm$ 0.03 <sup>b</sup>	1.12 $\pm$ 0.02 <sup>b</sup>
Angle of bulbus arteriosus	(°)	38.08 $\pm$ 1.00 <sup>a</sup>	32.96 $\pm$ 1.18 <sup>b</sup>	34.21 $\pm$ 1.35 <sup>ab</sup>	30.76 $\pm$ 1.16 <sup>b</sup>
<b>c. Gill morphometry (40/family/ploidy)</b>					
Whole body-weight	(g)	842 $\pm$ 26 <sup>a</sup>	560 $\pm$ 17 <sup>b</sup>	896 $\pm$ 46 <sup>a</sup>	810 $\pm$ 33 <sup>a</sup>
Total filament length	(mm/kg)	318 $\pm$ 7 <sup>a</sup>	463 $\pm$ 12 <sup>b</sup>	326 $\pm$ 18 <sup>ab</sup>	344 $\pm$ 8 <sup>ab</sup>
Arch surface area	(cm <sup>2</sup> /kg)	9.5 $\pm$ 0.2 <sup>a</sup>	12.4 $\pm$ 0.2 <sup>ab</sup>	10.1 $\pm$ 0.4 <sup>b</sup>	10.4 $\pm$ 0.3 <sup>b</sup>
Arch bone length	(cm/kg)	7.2 $\pm$ 0.2 <sup>a</sup>	10.0 $\pm$ 0.3 <sup>ab</sup>	7.4 $\pm$ 0.4 <sup>b</sup>	7.8 $\pm$ 0.3 <sup>b</sup>
Density of filament	(n/cm/kg)	22.4 $\pm$ 0.9 <sup>a</sup>	36.6 $\pm$ 1.6 <sup>a</sup>	20.5 $\pm$ 1.8 <sup>b</sup>	22.6 $\pm$ 1.5 <sup>b</sup>
Number of filament	(n)	110 $\pm$ 1	111 $\pm$ 1	108 $\pm$ 1	108 $\pm$ 1
<b>d. Prevalence of cataract based on macroscopic scoring (110/family/ploidy)</b>					
At least one eye $\geq$ 1	(%)	39.2 <sup>a</sup>	60.0 <sup>b</sup>	91.0 <sup>c</sup>	92.7 <sup>c</sup>
At least one eye $\geq$ 2	(%)	17.7 <sup>a</sup>	33.3 <sup>b</sup>	70.3 <sup>c</sup>	67.9 <sup>c</sup>
Both eyes $\geq$ 1	(%)	11.5 <sup>a</sup>	31.1 <sup>b</sup>	69.4 <sup>c</sup>	78.9 <sup>c</sup>
<b>e. Vertebrae (V) deformity (20/family/ploidy)</b>					
Total V number	(n)	58.1 $\pm$ 0.1 <sup>a</sup>	57.9 $\pm$ 0.2 <sup>a</sup>	57.1 $\pm$ 0.1 <sup>b</sup>	57.2 $\pm$ 0.1 <sup>b</sup>
Fish with $\geq$ 1 V deformed	(%)	65 <sup>ab</sup>	50 <sup>a</sup>	80 <sup>b</sup>	75 <sup>b</sup>
Fish with $\geq$ 2 V deformed	(%)	30 <sup>a</sup>	45 <sup>a</sup>	75 <sup>b</sup>	35 <sup>a</sup>
Fish with $\geq$ 3 V deformed	(%)	25 <sup>a</sup>	40 <sup>ab</sup>	60 <sup>b</sup>	30 <sup>a</sup>

### 3.3. Heart and gill morphometry

There was no effect of ploidy on the cardio-somatic index (CSI) and, within diploids only, F1 had a significantly lower CSI than F2 (Table 2b). One diploid (F1) and four triploids (40 heart assessed/family/ploidy) had a visibly deformed heart (clear rounded-shape, deformed bulbus arteriosus and split ventricule; data not shown). They were excluded from the dataset for morphometric analysis as outliers (Table 2b). There was a significant effect of ploidy on the heart height within F2 (triploid>diploid) but not on the heart width. There was a significant effect of family on the heart height within diploids (F1>F2) and on the heart width within both ploidies (F1>F2). The heart height/width ratio was always higher in triploids but this was significant within F2 only. The angle of bulbous arteriosus was more acute in triploids, but it was significantly more obtuse in F1 diploids compared to F2 diploids only (Table 2b).

Significant differences in BW occurred within the subsample used to assess gill morphology. As observed within the growth sample (Table 1b), F2 diploids had a significantly lower BW than all other experimental groups (Table 2c). The total filament length, second gill arch surface area (ASA) and arch bone length increased with BW when expressed in absolute values but decreased with BW when expressed relative to the fish BW. This is illustrated by the strong negative power relationship between the relative total length of filament and BW (Fig. 2a). The filament count did not vary with BW such that their relative density increased with BW. Using log-transformed data for linearity, BW was confirmed as a significant covariate ( $p<0.001$ , ANCOVA) for each parameter of gill morphology expressed relative to the fish BW (except for filament count). The adjusted means of the relative length of identified filaments (Fig. 2b) and of the total filaments (Table 2c) were significantly higher for F2 diploids than for all other experimental groups among which no differences occurred. There was a significant



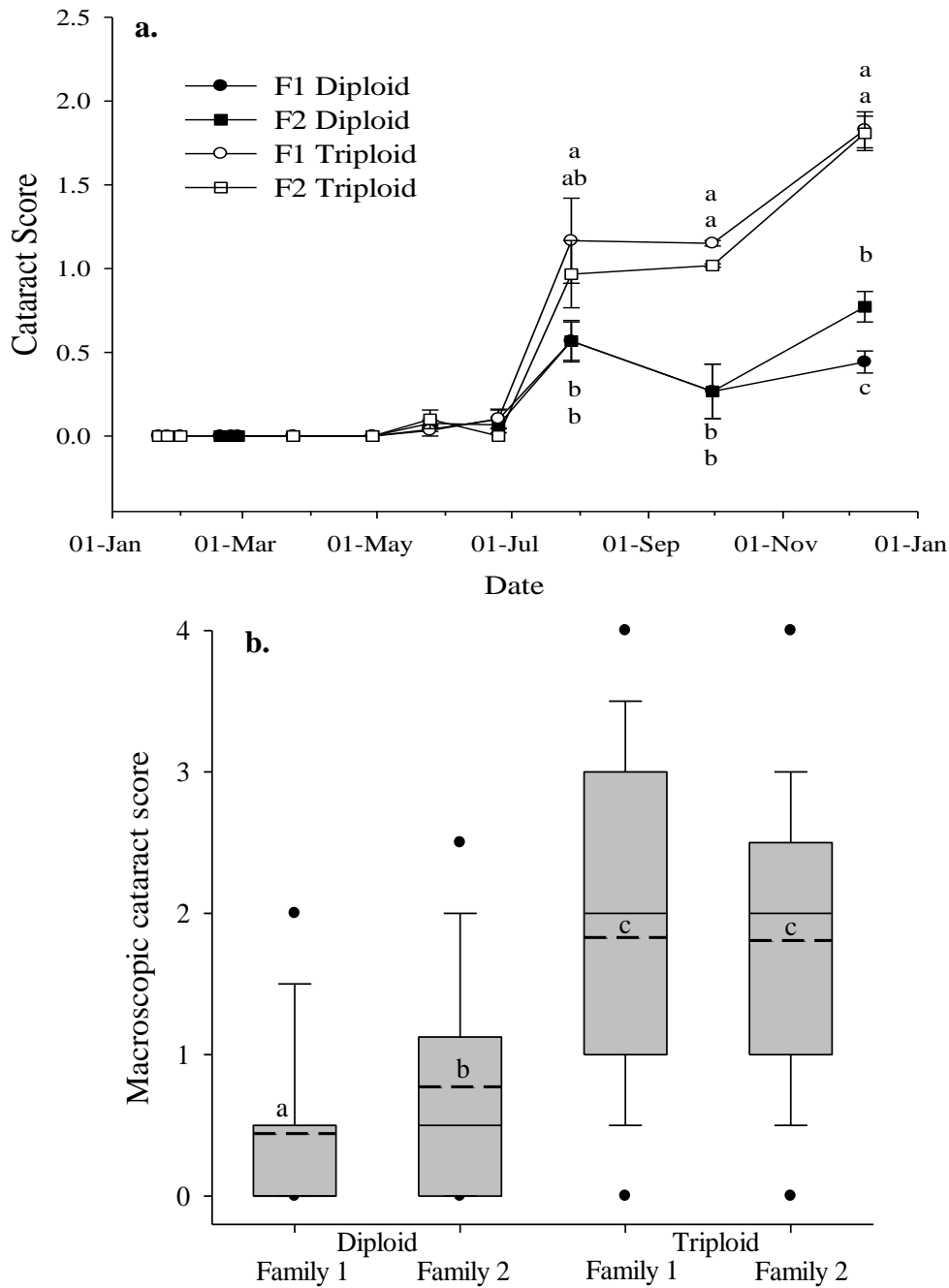
**Figure 2. a.** Relationship between total length of filaments (from the left-hand side, second gill arch, relative to whole body-weight) and whole body-weight (BW). The strong negative relationship among all experimental groups is highlighted by the strong regression coefficient of the power regression line of the whole experimental population. **b.** Individual filament length (from the left-hand side, second gill arch) relative to BW at the end of the first year at sea. The filament number 0 was defined as located on the tip of the arch bone curvature from which positive numbers were given towards the ventral-end and negative numbers towards the dorsal-end of the apparatus. Note: only the longest filament from both hemibranches was measured for any given location. Values are expressed as mean  $\pm$  SE ( $n = 2$ , 20 gill arches/family/ploidy). The relative length of individual filaments adjusted to BW was always significantly longer for F2 diploids than for any other groups (ANCOVA,  $p < 0.05$ ). F: family.

effect of ploidy on the adjusted means of the relative ASA and of the relative length of the arch bone within F1 only (triploids>diploids). The length of the arch bone was a significant covariate of the ASA (data not shown) and, adjusted to both BW and arch bone length, there were no differences in the mean ASA between experimental groups (data not shown). The total filament number did not vary with family, ploidy nor with BW which was not a significant covariate of this parameter. The adjusted mean of the relative density of filaments was significantly lower in triploids than in diploids within both families (Table 2c).

### **3.4. Ocular cataract**

Cataracts were not observed at the FW stage and appeared in all experimental groups from August, five to six months post-SWT (Fig. 3a). From August onward, both triploid families had a significantly higher mean cataract score than their sibling diploids. At the end of their first year at sea, the prevalence of individuals with one or both eyes affected by a macroscopic cataract score of at least 1 or 2 was significantly higher in triploids than in diploids within both families (Table 2d). There was no family effect in triploids, but within diploids, F1 had a significantly lower rate of macroscopic cataract (Table 2d; Fig. 3b).

Histological observations revealed that vacuolation of the lens fiber and epithelial proliferation (2 to 3 cells thickness) were common among the less affected fish. More severe cataracts were typically characterized by disruption of the lens fibers, formation of proteinaceous lakes with large areas of liquefaction and proliferation of the epithelium up to 5 cells thickness or more. Distribution of lesions was slightly different between ploidies (Table 3). The epithelium and anterior cortex were the main locations in diploids (macroscopic score 2 and 3) while the perinucleus and posterior cortex were more affected



**Figure 3.** (a.) Mean macroscopic cataract score in each family-ploidy group during their first year at sea. Values are expressed as mean  $\pm$  SE ( $n = 2, 110$  individuals/family/ploidy). Different superscripts denote significant differences between experimental groups within each sampling point (Kruskal Wallis Test with Dunn's Multiple Comparison post-hoc,  $p < 0.05$ ). (b.) Box-plot representation of the macroscopic cataract score in each family-ploidy group at the end of their first year at sea (Based on Wall and Bjerkås, 1999 scoring scale: Cataract score 0 = 0%, 1 = 1-10%; 2 = 10-50%; 3 = 50-75%, 4 = 75-100% lens cataract cover). The solid central line is the median, the box denotes 25<sup>th</sup> and 75<sup>th</sup> percentile, whiskers are 10<sup>th</sup> and 90<sup>th</sup> percentiles and the black circle symbol the 5<sup>th</sup> and 95<sup>th</sup> percentiles. The bold dashed line is the mean ( $n = 2, 110$  individuals/family/ploidy) shown with statistical differences between experimental groups as denoted by different letters (ANOVA,  $p < 0.05$ ).

at this stage in triploids. At an advanced stage of cataract observed in triploids only, the anterior cortex and the perinucleus were the most severely affected. There was no capsular involvement and the lens nucleus was only bulged in some of the most affected fish. Overall, the severity of the cataract in these discrete areas increased with the macroscopic cataract score.

**Table 3.** Relationship between macroscopic and microscopic cataract scores, in each family-ploidy group at the end of their first year at sea, highlighting the ocular areas affected by cataractous changes. Macroscopic scores based on the cataract covering of the lens diameter (Wall and Bjerkås, 1999). Microscopic scores based on a scale of 0 to 4 (0: absent, 1: slight, 2: moderate, 3: severe, 4: intense; scale modified from and ocular areas described by Wall and Richards, 1992). For each macroscopic score-ploidy group, the mean microscopic score was determined within each ocular area. The mean highest score is the average of the highest scores given to each fish in any area. Mean area scores higher than 1 are highlighted in bold and underlined when higher than 2.

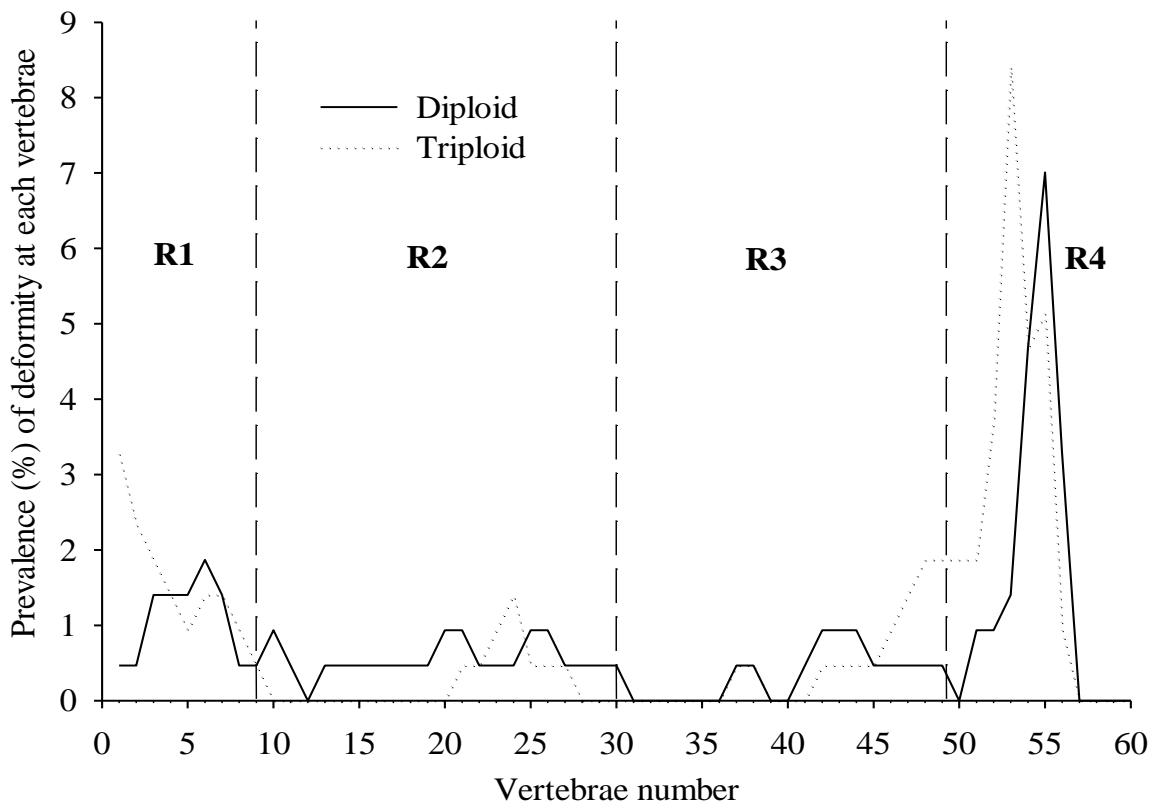
Macroscopic score	Diploid			Triploid			
	0	1	2 and 3	0	1	2 and 3	4
N assessed microscopically	5	5	5	3	5	8	5
<b>Mean microscopic scores within each ocular area</b>							
Capsule	0	0	0.2	0	0	0	0
Epithelium	0.4	0.6	<b>1.2</b>	0.3	0.6	<b>1.4</b>	<b>1.4</b>
Anterior cortex	0.2	1	<b>1.8</b>	0.3	0	<b>1.3</b>	<b><u>3</u></b>
Perinucleus	0.4	0.2	1	0.3	0	<b>1.5</b>	<b><u>3</u></b>
Nucleus	1	0.2	0	0	0.2	0.13	0.8
Posterior cortex	0	0.6	1	0.7	<b>1.6</b>	<b>1.8</b>	<b>1.6</b>
Mean highest score	1.2	1	2.2	0.7	2	2.4	3.4

### 3.5. Vertebral deformity

The total number of vertebrae was significantly affected by ploidy. Within both families, triploids had on average 1 vertebra less than their sibling diploids (Table 2e). The prevalence of individuals with at least one vertebra deformed was not affected by family (diploids: 57.5%, triploids: 77.5%) and was significantly higher in triploids within F2 only (Table 2e). The prevalence of individuals with at least 2 or 3 deformed



vertebrae was significantly higher in F1 triploids only and similar between F2 triploids, F1 and F2 diploids. Vertebrae 53 and 55 had the highest prevalence of deformities in triploids (8.4%) and diploids (7.0%) respectively (Fig. 4). In both ploidies, the most common type of deformity was a decrease in intervertebral space (~ 40%) which was observed at a higher prevalence in R1 for triploids and in R3 for diploids (Table 4). The second most common deformity was a one-sided compression (triploids: 26%; diploids: 15%) observed at the highest prevalence in R4. The tail fin region (R4) had the highest prevalence of deformities accounting for 44% (triploids) and 40% (diploids) of all deformities.



**Figure 4.** Prevalence (%) and localization of deformed vertebrae in diploid and triploid Atlantic salmon at the end of their first year at sea (based on the total number of deformed vertebrae observed within each ploidy; 98 and 117 deformed vertebrae in diploid and triploid respectively from 40 fish radiographed/ploidy). The different regions of the vertebral column are shown as follow: R1 (cranial trunk, V1→V8), R2 (caudal trunk, V9→V30), R3 (tail, V31→V49) and R4 (tail fin, V50→V57/58/59) with R: region and V: vertebrae.

**Table 4.** Prevalence, type and localization of vertebral deformity in diploid and triploid Atlantic salmon at the end of their first year at sea as characterized by radiology. Values are expressed as percentage of the total number of deformed vertebrae observed within each ploidy with n = 40 fish radiographed/ploidy. See Fig.4 for a description of the different vertebral column regions (R). InterVS: intervertebral. For each ploidy group, the two main type of deformity within each area or within the whole vertebral column are highlighted in bold.

	Diploid					Triploid				
	R1	R2	R3	R4	Total	R1	R2	R3	R4	Total
<b>Type of vertebral deformity (%)</b>										
Decreased InterVS	8	5	<b>14</b>	9	<b>36.7</b>	<b>19</b>	1	9	10	<b>39.3</b>
Homogeneous comp.	-	1	-	2	3.1	1	-	-	2	2.6
Comp. & decreased InterVS	5	-	-	-	5.1	3	-	-	3	6.0
Comp. without X structure	-	-	-	<b>13</b>	13.3	1	-	-	2	2.6
One-sided comp.	2	6	-	7	<b>15.3</b>	2	4	5	<b>15</b>	<b>25.6</b>
Comp. and fusion	-	3	-	-	3.1	4	-	2	-	6.0
Complete fusion	-	-	-	2	2.0	1	-	-	2	2.6
Fusion centre	4	8	-	2	14.3	1	3	-	-	4.3
Internal dorsal or ventral shift	-	3	-	4	7.1	-	-	-	11	11.1
Total (%)	19	27	14	40	100	31	9	16	44	100
Total (n)	19	26	14	39	98	36	10	19	52	117

#### 4. Discussion

This study showed that triploid Atlantic salmon post-smolts subjected to an accelerated smoltification regime can grow as well or better than diploid siblings and show equally good survival. The main potential problems observed in triploids were a higher prevalence of external deformities, radiological vertebral deformities and ocular cataracts, together with differences in heart morphometry. These are discussed with their likely origin, consequences and potentials for remediation.

A strong BW advantage was observed in triploids at the end of the FW-stage and the same pattern remained prevalent throughout the first year at sea. Triploids had a higher relative weight-gain (RWG; data not shown) and a lower SGR over the SW period. Comparison of these growth indicators was compromised by differences in BW at time of SWT and by the fact that triploids were transferred to sea earlier and under lower water temperatures. The TGC showed lower variations between ploidies and was,

compared to their sibling diploids, higher in F2 triploid (+5.3%) and only slightly lower in F1 triploid (-2.5%). Overall, triploids grew as well or better than diploids in SW. This is in accordance with O'Flynn (1997) and Oppedal *et al.* (2003), but in contrast with other studies showing similar (MacGeachy *et al.*; 1995; O'Flynn, 1997; Cotter *et al.*, 2002; Garner *et al.*, 2008) or slower (Whitler *et al.*, 1995; Shrimpton *et al.*, 2007) triploid growth in FW and slower (Whitler *et al.*, 1995) or similar (Cotter *et al.*, 2002) triploid growth in SW. The similar K found between ploidies over most of the sea rearing phase supports the hypothesis of Hyndman *et al.* (2003) stating that the effect of ploidy on cell size is likely less pronounced in elongated (muscle) cells where the relative nucleus volume is smaller than in spherical cells (blood). Alternatively, the higher growth by muscle fiber hypertrophy documented in triploids (Poontawee *et al.*, 2007) could compensate for the lower number of white muscle fiber per unit area (Suresh and Sheehan, 1998; Johnston *et al.*, 1999) and result in similar K between ploidies.

The high survival and lack of difference between ploidies and families are in accordance with earlier studies in SW (i.e. Oppedal *et al.*, 2003; Johnson *et al.*, 2004). Mortality rates in triploid salmon in SW are often within commercially acceptable levels, with the highest mortality during embryonic and larval development prior to first-feeding (Johnson *et al.*, 2004). Not previously reported, the timing of smoltification differed between ploidies in the current study. Triploids completed smoltification (assessed through seawater challenges and ATPase analyses) and were transferred to SW 4 weeks earlier than diploids. Transfer to SW during the "smolt-window" is key to later performances at sea of Atlantic salmon post-smolts (Mortensen and Damsgård, 1998). In this study, the low rate of failed triploid smolts (shown by high survivals) and the good growth of triploids were likely achieved by transferring each ploidy to SW

according to their respective developmental stage. Improper smoltification is indeed a likely cause of increased triploid mortality as suggested by Galbreath and Thorgaard (1995). This might explain results obtained in previous studies (poorer performance of triploid fish) where diploids and triploids were transferred simultaneously to sea (McCarthy *et al.*, 1996; O'Flynn *et al.*, 1997; Benfey, 2001). Our findings also suggest differential osmoregulatory capacity between ploidies at the cellular level. The difference in the timing of smoltification between ploidies requires further investigations and this must be considered in future studies when comparing the seawater performance of diploid and triploid Atlantic salmon.

Triploids are typically associated with a higher prevalence of external deformities. In this study up to 12 months post-SWT, 12% of the triploid population had visible deformities and diploids were seldom affected (< 5%). Within triploids, 89% of external deformities were slight lower jaw twisting or upper jaw shortening ("pughead") and virtually no externally apparent vertebral deformities (e.g. scoliosis and lordosis, Norway; McKay and Gjerde, 1986; Powell *et al.*, 2009), GFDS (Tasmania; Sadler *et al.*, 2001), nor operculae shortening (Tasmania, Sadler *et al.*, 2001) were observed. In diploids, external deformities were predominantly upper jaw shortenings that have been scarcely documented in previous studies. Besides the readily observable jaw deformities, triploids had a significantly higher lower jaw index (LJI) although this remained inconspicuous to the naked eye and would most likely not impair their homeostasis or marketability. Canadian and Tasmanian stocks are also predominantly affected by lower jaw deformities (O'Flynn *et al.*, 1997; Benfey, 2001; Sadler *et al.*, 2001) and it is of interest to note that Atlantic salmon reared in Australia were originally imported from Canada (River Philip, Nova Scotia; Ward *et al.*, 1994; Innes and Elliott, 2006). However, some studies reported no negative effects of triploidy on deformity in

Norwegian (Oppedal *et al.*, 2003) and Irish (Cotter *et al.*, 2002) stocks. These conflicting results are likely to evolve from the differences in genetic background, environmental conditions and diets in the different studies. Different strains should be assessed simultaneously in order to distinguish the effect of strain and environment on the comparative performance of diploid and triploid Atlantic salmon. A high prevalence of radiological deformity was observed in this study with more than 50% of diploids and 75% of triploids affected. Similar levels have previously been reported in fast growing underyearling triploid post-smolt (Fjellidal *et al.*, 2009a and b). However, vertebral deformities typically ranged between 7% and 17% both at the FW and SW stage in diploids (Sullivan *et al.*, 2007a and b; Fjellidal *et al.*, 2007a and 2009a). The higher incidence of deformities observed in the tail fin region (~40%) is in accordance with previous SW studies (Berg *et al.*, 2006) and compressions were shown to develop in this area after SWT (Fjellidal *et al.*, 2009a). Vertebral deformities were indeed mainly compression-related (diploids: 76.6%, triploids: 82.1%) and dominated by decreased intervertebral space and one-sided compression in both ploidies while complete fusion accounted for less than 3% of the cases. Asymmetrical compression may result in later fusion while a decrease in intervertebral space is commonly the earliest, less advanced sign of both compression (platyspondyly) and fusion (ankylosis; Witten *et al.*, 2006; 2009). The type (platyspondyly), localization (tail fin) and high prevalence of deformities observed in the present study suggest a combination of causative factors in the form of rapid growth, accelerated smoltification regime and rapid temperature increase at SWT (from ~2 °C in FW to ~7 °C in SW). Rapid growth was shown to correlate with lower vertebral mineral content resulting in a softer bone matrix more susceptible to compression-related deformities (Fjellidal *et al.*, 2007b). Smoltification is further concomitant with a rapid growth in length of the caudal vertebrae which might

increase the existing time-lag between the processes of osteoid deposition and mineralization for bone formation (Fjelldal *et al.*, 2005, 2006). Additionally, SWT induces a decrease in the vertebral mineral content which might drop below a critical level at an early SW stage leading to vertebral deformity (Fjelldal *et al.*, 2006). The strong positive temperature difference at SWT might have further enhanced both bone demineralization and growth toward a mechanical overload on the weaker tail fin vertebrae (Witten *et al.*, 2009). The highest prevalence of deformity was indeed observed in F1 triploid which was the fastest growing group with a high condition factor. Calcium (Ca) and phosphorous (P) are recognized as the most limiting bone minerals (Baeverfjord *et al.*, 1998; Fjelldal *et al.*, 2007b). Feeding a diet supplemented with P and Ca from SWT and for 4 months was successful at reducing the incidence of vertebral deformity from 73% to 37% as observed after one year at sea (Fjelldal *et al.*, 2009b). Not previously assessed, we cannot exclude a possible effect of ploidy on bone cell structure or cellular metabolism which could in turn alter osteoid deposition and bone mineralization. In addition, we cannot also exclude an effect of excessive temperature at an early life-stage which is recognized as a key causative factor in various teleosts (Wargelius *et al.*, 2005). In particular, vertebral count is set during early ontogeny by complex genetic-environmental interactions (Goin *et al.*, 2008) and was shown to be affected by extreme temperatures at this stage (Loken and Pedersen, 1996). A higher susceptibility of triploids to high water temperatures could have been responsible for their lower vertebral count (one less vertebra on average), a condition which has not previously been reported. It could also have arisen from a complete fusion and remodeling of two vertebrae into one at an early stage (“containment”; Witten *et al.*, 2006) which would not be detectable in on-grown fish.

Another widely reported problem in the North Atlantic farmed salmon industry is ocular cataracts (Midtlyng *et al.*, 1999). In the present study, macroscopic cataracts were observed from 6-month post-SWT onward. At the end of the trial, diploids showed a mild incidence of slight to moderate cataract while triploids showed a high incidence of mild to severe cataracts. Advanced cataracts impair vision and growth (Breck and Sveier, 2001) and could have reduced the growth performances of triploids in SW as previously shown in diploid stocks (Ersdal *et al.*, 2001; Breck *et al.*, 2003). Vacuolation, epithelial proliferation, lens fiber disruption and formation of proteinaceous lakes are consistent with irreversible cataracts, suggesting a protracted time of development of lesions in these fish. Among possible causative agents, defective ocular osmoregulation should be considered as similar lesions were previously reported in osmotic cataract from post-smolt Atlantic salmon (Bjerkås *et al.*, 2003). This is supported by the areas affected in this study which are also typical of cataracts with an osmotic origin (Breck and Sveier, 2001). Such disruption could occur from variations in water quality parameters such as salinity (Breck and Sveier, 2001) and pH (Bjerkås *et al.*, 2003), but also a range of other, more indirect, causative factors such as nutritional deficiencies (Hargis, 1991; Waagbø *et al.*, 2003), thermal variations (Bjerkås *et al.*, 2001; Tröbø *et al.*, 2010) and genetic factors (Wall and Richards, 1992) in line with our findings of different cataract rates across diploid families. In particular, a strong correlation between rapid growth and cataract is documented (Bjerkås *et al.*, 1996; Breck and Sveier, 2001) such that cataract outbreaks typically occur during periods of high water temperature and growth (Bjerkås *et al.*, 2001). The growth spurt experienced by all experimental groups in July/August was a likely predisposing factor to the outbreak observed. Interestingly, cataractogenesis can be reduced by feeding a diet properly balanced in pro- and anti-oxidant (Waagbø *et al.*, 2003) or supplemented

with histidine (His) which is found at low levels in vegetable protein meals (Breck *et al.*, 2003; Breck *et al.*, 2005a and b; Tröbke *et al.*, 2010). These authors showed that higher His tissue reserves increase the lens His and *N*-acetyl-histidine (NAH; His-derivative) content and its osmoregulatory capacity. Importantly, the susceptibility of different Atlantic salmon strains to low dietary His was demonstrated (Breck *et al.*, 2005a).

There was no difference between ploidies in cardio-somatic index (CSI). Discrepancies in heart morphometry between ploidies (height/width ratio, bulbus arteriosus alignment) show that triploid hearts were less rounded and more akin to wild-fish heart morphology (Poppe *et al.*, 2003). Their apparent better condition may arise from an increased cardiac workload (Poppe *et al.*, 2003), as observed and discussed in fish with a severe history of amoebic gill disease (Powell *et al.*, 2002). The higher cardiac activity of triploids could be a direct consequence of their lower oxygen (O<sub>2</sub>) carrying capacity, as demonstrated in triploid chinook salmon (*Oncorhynchus tshawytscha*), which were indeed shown to have the same O<sub>2</sub> consumption rate and therefore to compensate (Bernier, *et al.*, 2004). A higher basal cardiac workload could in turn explain the lower swimming endurance previously reported in triploids (Cotterell and Wardle (2004). Present knowledge on the effect of triploidy on hematological parameters was thoroughly discussed by these authors and the origin of different blood aerobic capacities is not clearly identified. In our study, no GFDS was observed such that the lower gill surface area reported in triploids (Sadler *et al.*, 2001) was not evident. The surface area of the second gill arch but not the total filament length was significantly higher in triploids and this could have been adaptative to their lower blood O<sub>2</sub>-carrying capacity. However, this was based on the overall arch perimeter and is not likely to reflect the true gill surface area since triploids had a significantly lower density



of filaments. An effect of ploidy on gill size/morphology was hypothesized as responsible for differences in recovery from exhaustive exercise (Hyndman *et al.*, 2003). Differences were observed in this study but remained inconclusive such that a thorough comparative assessment of the gill apparatus, including filament/lamellae morphometry and perfusion, water-blood diffusion distance and other parameters affecting respiratory gas transfer (Wells and Pinder, 1996; Perry, 1998; Flajšhans and Piačková, 2006) is required to estimate any morphological/cytological limitation or adaptation of the triploid gill apparatus.

A higher cardiac rate could also compensate for a reduced gill irrigation induced by a lower jaw deformity which was shown to restrict recovery from exhaustive swimming (Lijalad and Powell, 2009) and the same consequences can be expected from upper jaw deformity. However, lower jaw deformity was shown not to affect growth (O'Flynn, 1997). Similarly, fusion-related vertebral deformity (although compressions were observed in this study) reduced swimming speed and increased metabolic rate thereby reducing energy available for growth (Powell *et al.*, 2009). However, radiologically deformed fish showed no reduction in growth unlike fish showing external signs of vertebral deformities (Fjellidal *et al.*, 2007a). As indicated by Benfey (2001), fish with a jaw deformity cannot be sold head-on but this condition does not affect value-added processing. It is also likely that 1 or 2 vertebral compression would not adversely affect the fish homeostasis nor its marketability. Importantly, most deformities observed in the present study occurred in the tail fin region which would not affect filleting yield. However, these could impact on the welfare of triploid Atlantic salmon and must therefore be addressed before commercial implementation of triploidization.

Triploids from the Scottish strain tested in the present study performed as well or better than diploids in terms of growth and survival. The present study warrants and will facilitate further research aiming at adapting rearing practices to the needs of triploid stocks in order to improve their zootechnical performances and welfare. The value of different strains of Atlantic salmon as candidate founders of a triploid selective breeding program should also be tested. Knowledge on the origin of vertebral deformities and cataract outbreaks is rapidly expanding and prevention through parental selections, nutritional supplementation and improved husbandry practices were shown effective in diploids. It is envisaged that the same causative factors are involved in triploids and that similar improvements can be achieved making triploidization a promising strategy for a more sustainable salmon industry.

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**CHAPTER 7**

**SUMMARY OF FINDINGS**

## CHAPTER 7 SUMMARY OF FINDINGS

In this section, the main findings of each research manuscript are summarized and each study is schematically presented along the Atlantic salmon production cycle (Fig.1).

### **PAPER III** *Body size dimorphism of sea-reared Atlantic salmon (Salmo salar L.): Implications for the management of sexual maturation and harvest quality.*

- Body size dimorphisms between maturity cohorts are standard among discrete rearing units stocked with the same post-smolt stock. They can be modeled to estimate and selectively harvest a high proportion of maturing fish before any deteriorations of their flesh quality.
- Sexual size dimorphisms occur in immature Atlantic salmon with males exhibiting a significant body-weight advantage.
- Occurring dimorphisms alter the population structure and the apparent stock performances following weight-grading.

### **PAPER IV** *Seasonal variations in skin pigmentation and flesh quality of Atlantic salmon (Salmo salar L.): Implications for quality management.*

- A lack of purine pigment compounds is responsible for the distinctive lightness and yellowness of some immature Atlantic salmon.
- This altered phenotype does not reflect deteriorations in flesh quality and skin colouration. It can be reconditioned at a post-mortem stage by direct ice contact.
- Nuptial display also originates from a reduced level of purine pigments and is sexually dichromatic with nuptial male accumulating more skin carotenoids than female.
- The increase in total gonad carotenoids is higher in male than in female

### **PAPER V** *The use of continuous light to suppress pre-harvest sexual maturation in sea-reared Atlantic salmon (Salmo salar L.) can be reduced to a 4-month window.*

- The duration of continuous light exposure can be reduced by 30% without compromising its efficiency at suppressing sexual maturation.
- Females can commit toward maturation under a short-day window which should be considered as an open, photo-neutral stage, within the endogenous reproductive cycle.

**PAPER VI** *The potential of alternative lighting-systems to suppress pre-harvest sexual maturation of 1+ Atlantic salmon (*Salmo salar*) post-smolts reared in commercial sea cages.*

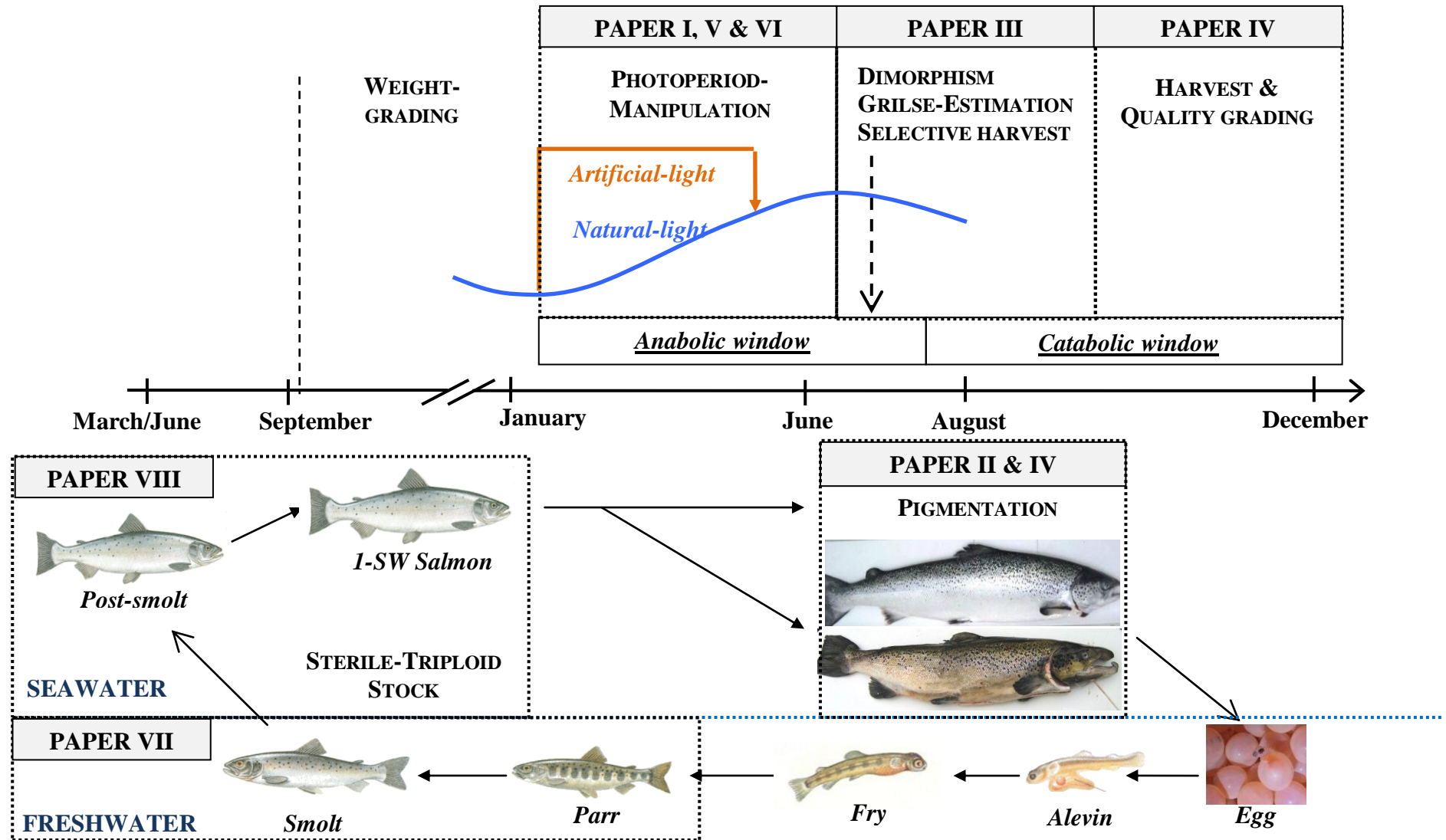
- Pre-harvest sexual maturation can be efficiently suppressed using alternative lighting-technologies and financial returns can be improved by deploying a lighting-strategy scaled in accordance to the data provided.
- The mean-irradiance (intensity) generated within a commercial sea-cage is inversely proportional to the suppression of nocturnal plasma melatonin and negatively correlates with the pen maturation rate. The nocturnal rise in plasma melatonin must be reduced to a non-significant 1.2-fold increase above day-time level to mask the circadian photoperiod cycle and suppress maturation.
- The running cost of photoperiod manipulation is negligible in comparison to value of the biomass that would otherwise mature. Photoperiod manipulation must aim at a complete suppression of maturation.

**PAPER VII** *Parr-smolt transformation in out-of-season triploid Atlantic salmon (*Salmo salar* L.).*

- Out-of-season triploid smolts can be produced using an accelerated "square-wave" photoperiod.
- Triploidization affected the smoltification pattern but had no detrimental effects on FW and early SW performance under both a S0+ and S1 regime.

**PAPER VIII** *Comparative seawater performance and deformity prevalence in out-of-season diploid and triploid Atlantic salmon (*Salmo salar*) post-smolts.*

- Survival and growth of out-of-season post-smolts is not affected by triploidization.
- Vertebral deformities and cataract outbreaks are the prime SW bottlenecks in triploid stocks and are likely to originate from nutritional deficiencies.



**Figure 1.** Schematic representation of the studies performed on the management of pre-harvest sexual maturation along the production-cycle of Atlantic salmon.

**CHAPTER 8**  
**GENERAL DISCUSSION**

## **CHAPTER 8 GENERAL DISCUSSION**

The general aim of this research project was to improve the management of sexual maturation in the Atlantic salmon on-growing industry with the view to optimizing financial output, production predictability, environmental respect, animal welfare and overall sustainability. To that end, two main approaches were taken. The first aimed at improving the panel of complementary practices currently deployed: weight-grading, harvest and post-harvest quality grading (Chapter 4) and photoperiod manipulation (Chapter 5). The second direction was to assess the feasibility of producing out-of-season sterile-triploid Atlantic salmon smolts and to determine their zootechnical performance both in FW and SW (Chapter 6). This second research area would constitute a distinct, single and comprehensive solution to the problem of sexual maturation but remains a longer-term perspective that is discussed separately.

### **1. Improvement of the current set of management practices**

Aiming at providing practical solutions that could be directly implemented within the industry, investigations into the current management practices tested readily applicable hypotheses or alternative practices using Atlantic salmon stocks of commercial origin and reared under full commercial management. The limitations inherent in such experimental conditions are addressed along with the wider implications of the present findings and the areas for further research identified.



### **1.1. Body-size dimorphism and selective harvest**

Our data revealed strong body-size dimorphisms in 2-SW Atlantic salmon thereby highlighting the need for a thorough investigation on their dynamic and origin. In this study, dimorphisms were assessed over time within a number of discrete harvest batches (within and across different sea-sites) previously subjected to size-grading reared in four distinct sea-sites. The time-course of the dimorphisms could not be described for any of the 2-SW Atlantic salmon population studied. Additionally, variations in environmental and husbandry parameters did not allow consideration of distinct sea-sites as replicates.

The limitations imposed by our experimental design also constituted the strength of the present studies. It allowed demonstration that body-size dimorphisms were standard across discrete rearing-units constituting a “rearing-cluster” as discussed in the corresponding chapter. This presents strong practical implications for large-scale, multi-site aquacultural operations. Further improvement of the proposed dimorphism-based management strategy can be foreseen. In particular, the industry would benefit greatly from accumulating strain-specific knowledge on dimorphic-growth patterns in order to facilitate maturation management over successive generations. The development of a real-time, on-site and non-invasive maturation detection kit (quantitative “drop” blood test of sex-steroids by immunochromatographic assays using lateral flow technology) would be a useful analytical tool within the proposed strategy in order to reduce the reliance on a model dataset. Maturation rate could be readily estimated within individual pens and the related dimorphism characterized simultaneously in order to define the most optimal, pen-specific, weight-grading strategy for the selective harvest of a high proportion of maturing fish. Over time, the acquired datasets would bring

valuable knowledge in the understanding and piloting of the plasticity of age and size at first maturity (Taranger *et al.*, 2010). It is acknowledged that weight-grading is actually done on body-width (“parallel” bars within the grader) and not on body-weight. Maturing fish were significantly heavier and also had a higher K than immature fish. Discrepancies in width between maturity cohorts would therefore be more pronounced than discrepancies in body-weight. Consequently, the actual proportion of maturing fish segregated during weight-grading would be higher than the rate of selective harvest estimated based on body-weight. In future studies, body-width should be measured when assessing the effect of body-size grading on cohort segregation (maturity and/or gender).

Describing the time-course of body-size dimorphisms between genders and maturity cohorts, within different commercial strains, over the whole-production cycle and according to environmental conditions and husbandry practices would have been of strong experimental and commercial interest and best performed using individually tagged fish reared in common (Imslad *et al.*, 1997; Saillant *et al.*, 2001). The physiological origin of such dimorphisms remains unclear (Fontaine *et al.*, 1997; Imslad *et al.*, 1997) and could not be elucidated in the present project. In future studies, key endocrine parameters describing the activity of the brain-pituitary-gonadal-axis (BPG, e.g. sex-steroids and gonadotropins hormones) and of the somatotropic-axis (e.g. growth-hormone releasing factor (GHRH), growth hormone (GH), somatostatin, insulin-like growth factor (IGF) and thyroid hormones (TH)) according to the gender should be assessed. These factors have previously been shown to be sexually dimorphic in teleost species (Patiño and Schreck, 1986; Margolis-nunno *et al.*, 1987; Fukada *et al.*, 2003; Mandiki *et al.*, 2004; Davies *et al.*, 2008; Rennie *et al.*, 2008) and should be characterized in relation to nutritional and metabolic factors (e.g. feed intake and

utilization, specific growth rate, resource allocation, metabolic rate) under controlled conditions (e.g. restricted or unrestricted feeding; temperature).

Knowledge on the origin of SSD could allow adaptation of rearing practices (feeding regime, light-manipulation) to minimize the occurrence of sexual-dimorphism leading to unwanted size-variation. This could emphasise the benefit of using monosex Atlantic salmon stock which could be further enhanced by adapting husbandry practices to the gender. For example, feeding tables and diet composition do not take into account gender specific requirements. It could be argued that the growth of the best performing gender is typically not optimized in mixed-sex stocks which could explain the lower condition factor of the male cohort which was systematically heavier (Chapter 4; Paper III). Dimorphism could also affect flesh quality as illustrated by the lower total flesh carotenoids measured in immature males (Chapter 4; Paper IV). Flesh carotenoid concentration decreases with feed intake (Rørvik *et al.*, 2010) such that males might require a higher level of dietary carotenoid to achieve similar retention. Similarly, this might highlight sex-specific micronutrient requirements.

The current management practice of weight-grading during the first winter at sea followed by harvesting of the best performing cohort ahead of the reproductive season, as advised by the RSPCA Freedom Food labeling scheme (RSPCA, 2010), acknowledges the practical significance of dimorphism between maturity cohorts. However, they remain poorly defined and empirically used hence the need for a better characterization of the genetic-environmental factors determining the somatic thresholds (i.e. body-size dimorphism) required to proceed through maturation. Body-size dimorphism related to sexual development might also occur within an immature population as a result of the life-long seasonal pattern of gonadal activity shown to occur in various teleost species (see Chapter 2; Paper I). Strong evidence supports the

photoperiodic synchronization of gonadal development in pre-pubertal Atlantic salmon post-smolts and the same mechanisms could be in place at the FW-stage. Understanding how early gonadal and somatic development correlates, e.g. in FW and according to the prevalent photoperiod, could allow manipulating the fish age and size at first maturity by promoting somatic growth under a photo-inhibited gonadal status.

To this end, the developing knowledge on the transduction of the photoperiodic signal (melatonin) to the reproductive cascade (primarily GnRH) through the KiSS/GPR54 system is of strong interest but knowledge remains limited to this day (Migaud *et al.*, 2010; Chapter 2; Paper I). In particular, the link between photoperiod signaling and the BPG-axis controlling reproduction remains to be found (Migaud *et al.*, 2010). In addition, the relation between the KiSS/GPR54 system and the somatotrophic axis remains poorly studied. It was recently demonstrated that IGF-I is an activator of the KiSS-1 gene in the prepubertal female rat (Hiney *et al.*, 2009). If confirmed, this would help to understand how the somatic and the reproductive axis together interact and determine the age and size at first reproduction. It must be acknowledged that understanding of the central mechanisms that control both maturation and growth is moving at a fast pace, thanks to the recent development of molecular tools. The next few years should bring a lot of exciting new knowledge in these specific fields. Atlantic salmon is among the best studied seasonally breeding teleosts and the review on the photoperiodic entrainment and developmental regulation of the annual reproductive cycle (Chapter 2; Paper I) could facilitate hypothesis testing and experimental design for future studies.

## 1.2. Skin colouration and post-harvest quality grading

The segregation of a high proportion of maturing fish at the end of the anabolic window in June/July (Chapter 4; Paper III) presents its full benefit from their superior flesh quality at this early stage of maturation (Chapter 4; Paper IV).

Nuptial display over the catabolic window of maturation is well known to reflect a deterioration in flesh quality at an advanced stage of sexual maturation and constitutes a poor quality attribute in itself (Michie *et al.*, 2001). However, skin colour is a complex trait affected by a variety of factors (see Chapter 2; Paper II). Knowledge on skin colour/flesh quality relationships remains limited and motivated the study presented in Chapter 4 (Paper IV). The “intermediary” nuptial display did not relate to sexual maturation over most of the catabolic window of maturation and did not reflect an altered or inferior flesh quality.

By quantifying three key pigments (carotenoids, melanin and purines) within the integument of the same individuals, we described for the first time the pigmentary origin of skin colouration in Atlantic salmon and, in fact, any teleosts. Purine pigments in particular were not previously identified and quantified in teleost integument using HPLC, although a previous study used chromatography and spectrophotometry during smoltification in Atlantic salmon (Johnston and Eales, 1967). However, the analytical method applied did not allow simultaneous detection of pteridine pigments which are likely to be present in the integument of Atlantic salmon as in other salmonids and temperate teleosts (Premdas and Eales, 1976a and b; Fuji *et al.*, 2000). The method we used has been modified from previously published techniques successful in the simultaneous determination of both pigment compounds (Hudon and Muir, 1996; Porcar *et al.*, 1996). It is suggested that a preliminary separation of the pteridine compounds (Andondonskaja-Renz and Zeitler, 1983; Bel *et al.*, 1997 for methods)

would be required due to the low concentration of pteridine compared to purine compounds in teleost skin.

The intermediary colour phenotype was shown not to reflect sexual development over the reproductive cycle (except at the onset of nuptial display in June/July). However, it originated, at least partly, from a lack of purine pigment which was also measured, to a larger extent, in fish displaying full nuptial colouration. The level of purine pigment is known to vary during parr-smolt transformation through physiological mechanisms involving thyroid hormones (Chua and Eales 1971; Premdas and Eales 1976b; Bjerkeng *et al.* 1999; Chapter 2; Paper II). The intermediary phenotype observed is more likely to originate from physiological factors related to desmoltification rather than being an adaptation to the surrounding light conditions (background adaptation and water clarity) which commonly involves an increase in melanin pigment (Sugimoto, 1993; 2002). It could be proposed that it reflects an alteration in osmoregulatory competence with possible welfare implications under rearing containment. Observed in immature fish, it could relate to a natural behavior of “dummy-run” migration toward the spawning ground (e.g. brackish estuary) as shown in immature cod and plaice (Rose, 1993; Arnold and Metcalfe, 1995; Chapter 2; Paper II). This hypothesis requires further investigation relating skin purine levels with gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and performance or welfare indicators. However, a major limitation to the analysis of purine pigment compounds is their low solubility and high concentration in teleost skin. A preliminary investigation validating the correlation between skin reflectance and the level of purine pigment in the skin of adult Atlantic salmon, as shown during the parr/smolt transformation (Haner *et al.* 1995; Ando *et al.* 2005) would therefore be of interest. It could ultimately allow the use of skin reflectance as a rapid and non-lethal indicator of welfare and desmoltification.

Since the intermediary phenotype was shown to present optimal flesh quality attributes, the possibility of reconditioning (improving) their skin colour towards a more silver-like appearance was tested with the view to facilitate quality grading and later product acceptance. The effect of ice of different sodium chloride (NaCl) concentrations was selected from previous studies showing that Na<sup>+</sup> induces chromatophore dispersion (De L. Castrucci, 1975; Mayo and Burton, 1998). Importantly, it could be easily applied within the industry as Na<sup>+</sup> is the prevalent seawater salt (Turekian, 1968). The variations in skin colouration observed within hours of treatment demonstrated a strong effect of storage conditions on melanophore migration and thereby colour expression. However, this was driven by ice contact rather than Na<sup>+</sup> content such that storage conditions maximizing ice contact must be provided to optimize external colouration. Further research should assess the effect of slush quality (e.g. blood concentration, water/ice ratio) during intermediary storage following slaughtering in advance of quality grading. These might include maximizing bleeding at the harvest station prior to storage and/or providing an intermediary storage on ice prior to quality grading. This could in turn favour the expression of standard colouration which could facilitate grading of the fish into the most appropriate market channel. Going further, it would be interesting from a retailer perspective to assess if colour changes observed following 30h of ice contact are fixed or if chromatophores retain their motile capacity at this later stage of the product life-cycle.

### **1.3. Suppression of maturation through photoperiod-manipulation**

Selective harvest (Chapter 4; Paper III) and post-harvest quality grading (Chapter 4; Paper IV) are two remediation measures mitigating the negative impacts of occurring sexual maturation. In comparison, photoperiod manipulation is a preventive

measure suppressing the occurrence of pre-harvest sexual maturation. Following decades of study (Bromage *et al.*, 2001), the photoperiodic synchronization and developmental regulation of reproduction in salmonids is overall well understood (Chapter 2; Paper I) such that the light-manipulation strategy applied within the industry are usually highly effective. However, the present strategy is to apply LL from the winter to the summer solstice (6-month) using high power, wide-spectrum lighting-systems which can be regarded as the safest option but could largely be improved.

Reducing energy use has positive financial and environmental implications and would minimize the risk of compromising the animal welfare (Migaud *et al.*, 2007a). To that aim, a reduction in the duration of LL-exposure (Chapter 5; Paper V) and the use of alternative lighting-technologies of lower-intensity with specific spectral output (Chapter 5; Paper VI) were clearly identified as two commercially relevant means of improvements.

Based on the current knowledge on Atlantic salmon (Chapter 2; Paper I) and avian species (Sharp, 1996; Hahn and MacDougall-Shackleton, 2007), it was hypothesized that an earlier switch from long-to-short day would return the previously photo-inhibited immature cohort to a photo-neutral stage. The immature cohort would therefore resume basal gonadal development with no effect on the population maturation rate at harvest. Results supported this hypothesis but are acknowledged as preliminary and require further confirmation before implementation across the industry due to variations in management practices, strain and geographical conditions. However, the observation of females initiating true vitellogenesis in October/November was not expected. It showed that basal gonadal activity was indeed resumed but that recruitment into maturation also occurred under short-days in some individuals as previously suggested (Taranger *et al.*, 1999; 2010). This highlights the complex



reproductive strategy in Atlantic salmon reviewed in Chapter 2 (Paper I). It also showed the potential risk for the immature cohort of reaching excessive gonadal development at harvest if the LL-window is terminated too early (e.g. mid-March) and/or if the stock is harvested later (e.g. January onwards). Our findings were based on histological evidence such that the switch of the immature cohort to a photo-neutral stage following LL-offset remains to be ascertained through a physiological assessment of the BPG-axis activity.

As compared to the windows of exposure, studies on the effect of light-properties on the entrainment of salmonid reproductive cycles are comparatively scarce. The effect of light-intensity was previously investigated in on-growing Atlantic salmon (Oppedal, 1997, 1999; Porter, 1999) but knowledge on the effect of light-spectral composition is restricted and often based on *ex vivo* trials (Migaud *et al.*, 2006; 2007a; Vera *et al.*, 2010).

Despite the recent development of alternative technologies and their potential benefit in aquaculture systems, these have not been used so far in the salmon industry. This is primarily due to uncertainties on their biological potency and commercial risks involved. Our experimental design (Chapter 5; Paper VI) used the established light-manipulation window to test new prototype lighting-systems close to commercial production. Despite the fact that each light-unit emitted different intensities and light-spectra (generated from distinct technologies), the same number of units were deployed per pen to test, in each treatment, a commercially applicable lighting-strategy. However, this experimental design did not allow differentiation of the respective effects of light-intensity and spectral composition, particularly in view of the preponderant effect of intensity (Oppedal *et al.*, 1997, 1999). Further research is clearly needed in this field that remains poorly studied but highly relevant to various aquacultural species. In

particular, specific wavelengths might have differential effects on the inhibition of maturation, stimulation of growth, and stress response (Migaud *et al.*, 2007a). In particular, it would be interesting to determine if the threshold intensities that induce the biological effects compromise to any extent the animal welfare. Finally, different light-spectra might have an effect on fish skin colouration through mechanisms of background adaptation and in turn affect post harvest quality grading and product marketability. However, such effects are not expected to be particularly significant considering that fish reared under LL from January to June, May or April are harvested over the second half of the year thereby experiencing NL for a few months before harvest.

It must also be acknowledged that some of our trials (Chapter 5; Paper VI, Trial 2 and 3) could not include a control treatment (NP) due to the inherent risk of high maturation rate for the industrial partner. The possibility to selectively harvest a high proportion of maturing fish as a superior quality product opens the possibility for further risk taking at a commercial scale with regards to light-manipulation. This could motivate testing further reduced LL-windows (e.g. from early-January to end-March) using alternative technologies and NP controls.

Organic production of Atlantic salmon typically relies on local strains and does not allow light-manipulation to control pre-harvest maturation. The Soil Association (SA) organic standards for aquaculture producers (Soil Association, 2009) holds a prerequisite of minimizing environmental impacts, stress, disease incidence and use of veterinary products while ensuring for the stock the five freedoms from malnutrition and hunger, physical discomfort, injury and disease, fear and distress and unnecessary restriction of behaviour. Sexual maturation compromises all these requirements and arguably from the onset of the anabolic window of maturation. The negative impacts of

light-manipulation are, in comparison, to increase the farm energy inputs with inherent environmental impacts and possibly to negatively affect the stock welfare. However, impacts on welfare indicators have only been shown at specific wavelengths provided at high intensity and, so far, appeared to have no chronic but rather transient and mild effect on stress factors (increase in plasma cortisol and glucose level < 24h; Migaud *et al.*, 2007a). The possibility of reducing the intensity and duration of LL-exposure and to select specific-wavelength while achieving the highly beneficial effect (in term of welfare) of suppressing pre-harvest maturation could allow re-evaluating the use of light-manipulation for organic production. Additionally, more diffuse lighting-systems as compared to the point source metal-halide currently used, such as the 'green' hot cathode tested, provide a more consistent light field and reduce the occurrence of areas of high irradiance within the rearing environment. A thorough "cost-benefit" analysis of light-manipulation/sexual maturation would possibly demonstrate the net benefit of the improved lighting-strategies presently tested in term of animal welfare and environmental impacts.

## **2. Sterilization**

Despite the possibility of improving the current set of management strategies to address the problem of early maturation (Paper III, IV, V and VI), they remain complementary, hold a financial and environmental cost, require a degree of expertise for optimal implementation and cannot be fully reliable. Sterilization by triploidization is the only reliable means of preventing the negative impact of domesticated escapees on wild Atlantic salmon stocks and the propagation of non-native salmon (Fleming *et al.*, 1996; Piferrer *et al.*, 2009).

## 2.1. Production of triploid smolts out-of-season

Out-of-season smolt production is a vital component of the salmon industry in order to achieve year-round stocking of sea-sites and supply of market size fish. Poor smoltification has been suggested to be a reason for the reduced SW-performance of triploids (Withler *et al.*, 1995). The possibility of producing out-of-season triploid smolts using an accelerated photoperiod (Chapter 6; Paper VII) is a prerequisite for the industry and has not been previously investigated in Atlantic salmon.

The performance of the triploid cohort produced under an accelerated photoperiod-regime was higher than expected. In particular, their higher growth rate might have contributed to their low rate of failed smolt at sea. However, it must be recognized that an accelerated thermo-regime was not used due to limitation within our experimental facilities and this should be assessed as required when producing truly out-of-season, i.e. autumn, smolts.

Besides the successful production of out-of-season triploid smolts, our study also provided insight into the process and assessment of smoltification in both triploids and diploids. In particular, an absolute threshold of gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was confirmed as unlikely to be the most appropriate indicator of smoltification. The growth of the gill apparatus was shown not to be proportional to the somatic growth (isometric) but negatively allometric in 1 to 10g fry (Wells and Pinders, 1996) and a similar relationship was observed in SW post-smolts (Chapter 6; Paper VIII). In this respect, smaller smolts would have a greater area/volume ratio (Handeland and Stefansson; 2001) hence a lower gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity to achieve the same osmoregulatory ability (Chapter 6; Paper VII). Further research should address BW as a covariate of gill surface area and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity during parr-smolt transformation in order to make this indicator, widely used both experimentally and commercially, fully

meaningful and reliable. The different patterns of triploid smoltification produced both under an S0+ (earlier time, different size thresholds) and S1 (same time, lower growth rate) regime warrant the need to adapt the timing of SWT to the ploidy in order to maintain homeostasis. This could in itself illustrate the increasing consensus that triploids should be treated as a “new species” (Benfey, 2001) and is likely to facilitate proper smoltification of triploid stocks in future studies and hence to assess their true performances.

## **2.2. Seawater performance of out-of-season triploid smolts**

Performance in SW is ultimately key to the overall commercial value of a given Atlantic salmon stock. Low survival (e.g. Cotter *et al.*, 2002), high deformity prevalence (e.g. Sadler *et al.*, 2001) and high cataract rate (Wall and Richards, 1992) have previously but not systematically (Oppedal *et al.*, 2003) been documented in triploid stocks. The performance of out-of-season post-smolt in SW (Chapter 6; Paper VIII) is promising with vertebral deformities and cataract prevalence being the main issues unveiled. These are likely to be remediated by adapting husbandry practices to the particular needs of triploids in terms of nutritional requirements in particular, but also by selecting the most appropriate broodstock to produce more robust triploid stocks. Of significance was the mild prevalence of vertebral deformities and cataracts detected in diploid stocks. This would suggest a high sensitivity of out-of-season diploid smolts such that they could also benefit from nutritionally enriched diets as discussed in the corresponding chapter. This remains hypothetical as confirmation would have required comparing S0+ to S1 cohort which could not be done in the present study.

Overall, this study clearly demonstrated the potential of SW triploid stock to achieve excellent performance in the future. Besides the factors mentioned in the

corresponding chapter, performance of mixed-sex and all-female stocks should be compared. Triploid males could perform better than triploid females as shown in diploids (Chapter 4; Paper III). Furthermore, triploid males that are sterile still undergo gonadal development such that they are likely to show a “growth spurt” related to maturation (Oppedal *et al.*, 2003). Conversely, female growth could be reduced due to their physiological sterility, which is, however, a key attribute when aiming at restricting wild-domesticated fish interactions. In accordance, a cost-benefit of all-female versus mixed sex stocks is an important aspect and this should consider both production and ecological aspects. Going further, a solution could be to use all-female stocks in order to optimize ecological benefit and to optimize growth through light-manipulation but this photic stimulation of growth remains to be studied in triploids. Further commercial research on triploid Atlantic salmon would be best performed in selected cage facilities which offer different biotic and abiotic environmental conditions to tanks (e.g. water flow, O<sub>2</sub> levels, parameters fluctuation, stressful events). A key disadvantage of triploid stocks is their reduced performance under suboptimal conditions (Benfey, 2010). This could come from heart deformities although the low number of individuals assessed in the study requires further investigation. In that sense, our study also showed the need for a better description of the effect of triploidy on the gill apparatus, oxygen consumption and cardiac workload. Importantly, more fundamental research on the cytology, haematology, physiology, endocrinology, immunology and morpho-anatomy of triploid fish (Tiwarly *et al.*, 2004; Maxime, 2007; Benfey, 2010) is essential for the domestication of triploid stocks. One key area of concern that needs investigation is disease resistance.

**Conclusion**

The present research project provides means to optimize the current set of management practices presently applied to manage sexual maturation within the Atlantic salmon on-growing industry: light-manipulation, weight-grading, harvest, and quality grading. Proposed improvements have the potential to increase biomass and financial output, production predictability, environmental respect and animal welfare and will allow standardization of the overall control of pre-harvest sexual maturation. Their implementation provides a comprehensive strategy likely to favour a sustainable expansion of the Atlantic salmon industry. It is acknowledged that each practice remains complementary to one-another. This project also provided insight into the smoltification of triploid Atlantic salmon and characterized key bottlenecks, their potential origin and future research required to make the use of sterile-triploid stocks a commercial reality in the longer-term.

Besides their commercial implications, the results obtained improve our understanding on fundamental aspects of salmonid physiology such as the entrainment of the annual reproductive cycle, the expression of skin colouration and smoltification. By providing background knowledge and methodology, present findings will facilitate further research of commercial and more fundamental interests such as light-perception and its transduction to the reproductive cascade and interactions with somatic determinants.

Pre-harvest sexual maturation is a widespread problem among aquacultural finfish species. Various farmed species exhibit body-size dimorphisms, strong photoperiodic entrainment of reproduction and skin colouration is often key to their marketability. The results and methods presented are also likely to find applications in other aquacultural species and systems in which similar problems are to be faced.

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**PUBLICATIONS AND  
CONFERENCES ATTENDED**

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### PUBLICATIONS

- Leclercq, E.**, Taylor, J.F. and Migaud, H. Photoperiodic synchronisation and developmental regulation of the annual reproductive cycle in salmonids. **In preparation.**
- Leclercq, E.**, Taylor, J.F. and Migaud, H. (2010) Morphological skin colour changes in teleosts. *Fish and Fisheries* **11**, 159-193.
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- Taylor, J.F., **Leclercq, E.**, Preston, A.C., Guy, D. and Migaud, H. Parr-smolt transformation in out-of-season triploid Atlantic salmon (*Salmo salar*). *Aquaculture*, **In Press.**
- Leclercq, E.**, Taylor, J.F., Fison, D., Fjellidal, P.G., Diez-Padrisa, M., Hansen, T. and Migaud, H. (2011) Comparative seawater performance and deformity prevalence in out-of-season diploid and triploid Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology* **158**, 116-125.

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- Leclercq, E.**, Taylor, J.F. and Migaud, H. (2009) Management of sexual maturation in on-growing Atlantic salmon (*Salmo salar*): Development of on-farm tools. *Nordic Workshop for PhD students (NoWPaS)*, 19-22 March 2009, Southampton, UK. **Oral presentation.**
- Leclercq, E.**, Taylor, J.F. and Migaud, H. (2009) Management of sexual maturation in on-growing Atlantic salmon (*Salmo salar*): Development of on-farm tools. *Post-graduate Research Conference*, University of Stirling, 5 May 2009, Stirling, UK. **Oral presentation.**
- Leclercq, E.**, Taylor, J.F. and Migaud, H. (2009) Management of sexual maturation in on-growing Atlantic salmon (*Salmo salar*): Development of on-farm tools. *Aquaculture Europe*, 14-17 August, 2009, Trondheim, Norway. **Oral presentation.**
- Taylor J.F., **Leclercq E.**, Preston A.C., Guy D., and Migaud H. (2009) Parr-smolt transformation in out-of-season triploid Atlantic salmon (*Salmo salar*). *International Workshop on Smoltification*, Oregon State University, 20-24 September 2009, OR, USA. **Contribution.**
- Leclercq, E.**, Taylor, J.F. and Migaud, H. (2010) Light-manipulation strategy for out-of-season smolt production in freshwater loch-system and management of sexual maturation during seawater on-growing. *Seawater and Freshwater communications forum, Marine Harvest (Scotland)<sup>Ltd</sup>*, 27 January 2010, Aviemore, Scotland, UK. **Oral presentation.**
- Leclercq, E.**, Taylor, J.F. and Migaud, H. (2010) The potential of alternative lighting-systems and shorter window to control sexual maturation of sea-reared Atlantic salmon, *Salmo salar*. *Aquaculture Europe*, 5-8 October, 2010, Porto, Portugal. **Oral presentation.**
- Taylor J.F., **Leclercq E.**, Guy, D. and Migaud H. (2010) Family-ploidy interactions during freshwater grow-out and smoltification in Atlantic salmon. *Aquaculture Europe*, 5-8 October, 2010, Porto, Portugal. **Contribution.**