Research and development of triploid brown trout *Salmo trutta* (Linnaeus, 1758) for use in aquaculture and fisheries management.

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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

Freshwater sport fisheries contribute substantially to the economies of England and Wales. However, many trout fisheries rely partly or entirely on stocking farmed trout to maintain catches within freshwater fisheries. Farmed trout often differ genetically from their wild counterparts and wild trout could be at risk of reduced fitness due to interbreeding or competition with farmed fish. Therefore, to protect remaining wild brown trout (*Salmo trutta L*) populations and as a conservation measure, stocking policy has changed. Legislation introduced by the Environment Agency (EA, 2009) will now only give consent to stocking of rivers and some stillwaters with sterile, all-female triploid brown trout. There are reliable triploidy induction protocols for some other commercially important salmonid species however; there is limited knowledge on triploid induction in brown trout. Previously, triploid brown trout have been produced by heat shocks although reduced survivals were obtained suggesting that an optimised heat shock had not been identified, or that heat shock gives less consistent success than hydrostatic pressure shock (HP), which is now recognised as a more reliable technique to produce triploid fish. Thus the overall aim of this thesis was to conduct novel research to support the aquaculture and freshwater fisheries sector within the United Kingdom by optimising the production and furthering the knowledge of triploid brown trout.

Firstly, this PhD project investigated an optimised triploidy induction protocol using hydrostatic pressure (Chapter 2). In order to produce an optimised hydrostatic pressure induction protocol three experiments were conducted to (1) determine the optimal timing of HP shock application post-fertilisation, (2) define optimal pressure intensity and duration of the HP shock and (3) study the effect of temperature (6-12 °C) on triploid yields. Results indicated high survival to yolk sac absorption stage

(69.2 - 93.6 %) and high triploid yields (82.5 - 100 %) from the range of treatments applied. Furthermore, no significant differences in triploid rates were shown when shock timings and durations were adjusted according to the temperature used. In all treatments deformity prevalence remained low during incubation (<1.8 %) up to yolk sac absorption (~550 degree days post hatch). Overall, this study indicated that the optimised pressure shock for the induction of triploidy in brown trout delivering high survival and 100 % triploid rate (a prerequisite to brown trout restocking) is a shock with a magnitude of 689 Bar applied at 300 Centigrade Temperature Minutes (CTM) for 50 CTM duration. Regarding the assessment of triploid status, the second experimental chapter tested the accuracy and efficacy of three ploidy verification techniques (Chapter 3). Techniques studied were erythrocyte nuclei measurements (Image analysis), flow cytometry (Becton Dickinson Facscalibur flow cytometer) and DNA profiling (22 polymorphic microsatellite loci) to assess the effectiveness of triploidy induction in brown trout. Results indicated the validity of using erythrocyte indices major nuclear axis measurements, flow cytometric DNA distributions expressed as relative fluorescence (FL2-Area), and polymorphic microsatellite loci (Ssa410UOS, SSa197, Str2 and SsaD48) for assessing ploidy status in brown trout. Accuracy of each technique was assessed and indicated that all techniques correctly identified ploidy level indicating 100 % triploid rate for that commercial batch of brown trout. These techniques may be utilised within aquaculture and freshwater fisheries to ensure compliance with the legislation introduced by the EA.

As a result of the legislation introduced by the Environment Agency triploid brown trout will freely interact with diploid trout therefore there is a need to assess feeding response and behavioural differences between diploid and triploid trout prior to release. Therefore, in the third experimental chapter (Chapter 4) diploid and

triploid brown trout were acclimated for six weeks on two feeding regimes (floating/sinking pellet). Thereafter, aggression and surface feeding response was compared between pairs of all diploid, diploid and triploid and all triploid brown trout in a semi natural stream (flume). In each pairwise matching, fish of similar size were placed in allopatry and rank determined by the total number of aggressive interactions initiated. Dominant individuals initiated more aggression than subordinates, spent more time defending a territory and positioned themselves closer to the food source (*Gammarus pulex*) whereas subordinates occupied the peripheries. When ploidy was considered, diploid trout were more aggressive than triploid, and dominated their siblings when placed in pairwise matchings. However, surface feeding did not differ statistically between ploidy irrespective of feeding regime. Triploids adopted a sneak feeding strategy while diploids expended more time defending a territory. In addition, an assessment of whether triploids exhibited a similar social dominance to diploids when placed in allopatry was conducted. Although aggression was lower in triploid pairs than in the diploid/triploid pairs, a dominance hierarchy was observed between individuals of the same ploidy. Dominant triploid fish were more aggressive and consumed more feed items than subordinate individuals. Subordinate fish displayed a darker colour index than dominant fish suggesting increased stress levels. However, dominant triploid fish seemed more tolerant of subordinate individuals and did not display the same degree of invasive aggression as observed in the diploid/diploid or diploid/triploid matchings. These novel findings suggest that sterile triploid brown trout feed similarly but are less aggressive than diploid trout and therefore may provide freshwater fishery managers an alternative to stocking diploid brown trout.

In addition to research at the applied level in triploid brown trout, this thesis also examined the fundamental physiological effects of ploidy in response to temperature regime. Triploid salmonids have been shown to differ in their tolerance to environmental temperature. Therefore the fourth experimental chapter (Chapter 5) investigated whether temperature tolerance affected feed intake and exercise recovery. Diploid and triploid brown trout were exposed to an incremental temperature challenge (10 and 19 °C) and subsequent survival and feed intake rates were monitored. Triploids took longer to acclimate to the increase in temperature however feed intake were significantly greater in triploids at high temperature. In a follow on study, we investigated post-exercise recovery processes under each temperature regime (10 and 19 °C). Exhaustion was induced by 10 minutes of forced swimming, with subsequent haematological responses measured to determine the magnitude of recovery from exercise. Plasma parameters (alkaline phosphatase, aspartate aminotransferase, calcium, cholesterol, triglycerides, phosphorous, total protein, lactate, glucose, pH, magnesium, osmolality, potassium, sodium, chloride, lactate dehydrogenase) were measured for each ploidy. Basal samples were taken prior to exercise and then at: 1; 4, and 24 hours post-exercise. Contrary to previous studies, there was no triploid mortality during or after the exercise at either temperature. Although diploid and triploid brown trout responded metabolically to the exercise, the magnitude of the response was affected by ploidy and temperature. In particular, triploids had higher levels of plasma lactate, osmolality, and lower pH than diploids at 1 hour post exhaustive exercise. By 4 hours post-exercise plasma parameters analysed had returned to near basal levels. It was evident that the magnitude of the physiological disturbance post-exercise was greater in triploids than

diploids at 19 °C. This may have implications where catch and release is practiced on freshwater fisheries.

Overall, this work aimed to develop and/or refine current industry induction and assessment protocols while better understand the behaviour and physiology of diploid and triploid brown trout. The knowledge gained from this work provides aquaculture and freshwater fisheries with an optimised protocol, which delivers 100 % triploid rates and profitability without compromising farmed trout welfare, thus ultimately leading towards a more sustainable brown trout industry within the United Kingdom.

Keywords: *Salmo trutta* L, triploid, pressure shock, ploidy verification, triploid yield, behaviour, temperature, exhaustive exercise, deformity, radiology.

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LIST OF ABREVIATIONS

17α MTH 17 alpha methyl testosterone

AF3N All female triploid

ALP Alkaline phosphatase

ANOVA Analysis of variance

ASTA Aspartate aminotransferase

CTM Centigrade Temperature Minutes

DAPI 4', 6-diamidino-2-phenylindole

DD Degree Days

DNA Deoxyribonucleic acid

E2 17β-estradiol

EIFAC European Inland Fisheries Advisory Commission

ENL Erythrocyte Nuclear Length

EPOC Excess post-exercise oxygen consumption

FACS Fluorescence activated cell sorting

FC Flow cytometry

FCI First Cleavage Interval

FSC Forward scatter

GFD Gill filament disease

GLM General linear model

HP Hydrostatic pressure

Hb-O_2 Hemoglobin-oxygen

IgfI Insulin-like growth factor I

LDH Lactose dehydrogenase

L_f Fork length

LJD Lower jaw deformity

LL Continuous light

LN Natural logarithm

MPF Minutes Post fertilisation

MtDNA Mitochondrial DNA

NOR Nucleolar-organising region

PI Propidium Iodide

Psi Pounds per square inch

RBC Red blood cell

SNP Simulated Natural Photoperiod

SSC Side scatter

TGFSG Trout and Grayling Fisheries Strategy Group

YSA Yolk sack absorption

CHAPTER 1. GENERAL INTRODUCTION

1.1 Brown trout overview and life history

The natural distribution of brown trout extends from the Arctic ocean of northern Norway and the White Sea area of Russia, south to the Atlas mountain region of North Africa, and from Iceland in the west to the Aral Sea drainage of Afghanistan and Pakistan in the East (Behnke, 1986; Hamilton et al., 1989). Brown trout are a biologically and ecologically diverse species, with resident and anadromous forms existing from 42° latitude northwards (Ferguson, 1989). Brown trout are highly polymorphic and different populations vary considerably in their life histories. In some populations resident forms live their entire life in freshwater and spawn in smaller tributaries in the autumn. In other populations, only juveniles remain in freshwater while older trout migrate downstream to the nearest lake (lake-trout), estuary (estuarine or slob trout) or sea (sea trout) (Elliott, 1989). Sea trout (anadromous) migrate to the sea from rivers to feed before returning to mature and spawn in their native rivers. The strong homing instinct in brown trout guides them towards their natal river using the olfactory gland (Halvorsen & Stabell, 1990). The migratory behaviour trait has been shown to be highly heritable, while influenced by environmental changes in food availability, temperature, and water velocity (Jonsson and Jonsson, 2002). Both forms can co-exist and have been shown to interbreed (Hindar 1991), with little genetic differences shown between the two forms (Charles et al., 2005). However, the precise mechanisms and genetic influences underlying these two life histories are still not fully understood.

Brown trout are adapted to feeding on a wide range of benthos, zooplankton, insects and fish. The diverse diet has allowed survival in isolated upland waters,

where seasonality of diet availability is largely limited to the spring and summer months when temperature increments encourage the emergence of prey items. Diet varies with age, geographic location, environmental characteristics, and in many waters brown trout are the only naturally occurring primary predator (Laikre, 1999). In addition, brown trout interact ecologically with other species and are required for the continuation of freshwater molluscan life cycle. For example, the reproductive cycle of the freshwater pearl mussel (*Margaritifera margaritifera*) is dependent on the brown trout, as the larvae of the mussels inhabit the gills of trout and Atlantic salmon (*Salmo salar* L.) (Laikre, 1999).

1.2 Phylogeny structure and genetic variation.

The brown trout has a complex genetic structure and differentiation, including subspecies and sympatric isolated populations throughout its distribution (Ferguson, 1989; Bouza et al., 2001; Presa et al., 2002). It is a highly polymorphic species that lives in a variety of aquatic ecosystems, including lakes, streams and the ocean (Charles et al., 2006). It is the most widely distributed teleost in the Paleartic region, native to Europe, North Africa and North-West Asia (Bernatchez, 2001, Ferguson, 2004).

The development of molecular genetics, in particular laboratory techniques to accumulate population data has allowed detailed studies of brown trout lineages. In particular, electrophoresis of allelic variants at protein coding loci (allozymes) has been extensively used (e.g. Aebersold et al., 1987; Utter et al., 1987). More recently, DNA markers, such as maternally inherited mitochondrial DNA (mtDNA) and mini- and microsatellite DNA have been utilised (Bernatchez et al. 1992; Prodohl et al. 1997; Hansen et al. 2000). From this, five major brown trout evolutionary lineages

have been defined according to the geographic region in which they inhabit. These include the Atlantic (AT); Adriatic (AD); Danubian (DA); Mediterranean (ME) associated with the main European drainage basins. The Mormoratus lineage (MA) is distinguished by its phenotype and endemic distribution to the Adriatic drainage (Bernatchez, 1992). It is characterised by a marbled appearance and behavioural differences such as shifted spawning period compared to other lineages. As a result some authors class the MA lineage or marble trout as a subspecies (*Salmo trutta marmoratus;* Giuffra et al., 1994) or a distinct species (*Salmo marmoratus;* Berrebi et al., 2000; Meraner et al., 2010).

The evolutionary lineages have evolved in geographic isolation during the Pleistocene (circa. 13,000 $_{B.P.}$) and have remained largely allopatric since then (Bernatchez, 2001). There have been eight major glacial and interglacial periods, which resulted in large areas of Britain and Ireland covered in ice, with two periods of freshwater isolation during each glacial cycle. It is during these periods in which geographic isolation and divergent evolution of brown trout occurred (Hamilton et al., 1989). As a result of extensive lowering of the sea, land mass was considerably extended which may have provided refuges for trout, which today are marine environments (McKeown et al., 2010). The brown trout has the ability to be anadromous, and these refuges would have allowed for rapid colonisation by sea-run brown trout (McKeown et al., 2010). It has been suggested that most if not all of the native brown trout in Britain and Ireland, irrespective of current life history, colonised as a result of anadromy (Ferguson, 2006).

The colonisation of brown trout in Britain and Ireland was from five distinct glacial refuges including: South of England, Western France, East of the Baltic Sea, Western Ireland, Celtic Sea and North Sea (McKeown et al., 2010). As a result,

anadromy has allowed continued gene flow between populations in some adjacent rivers. Some inbreeding of lineages has occurred, resulting in mosaic patterns of genetic diversity while in others distinct lines remain resulting in sympatric reproductively isolated populations (McKeown et al., 2010). Wide ranging genetic diversity is found in populations of brown trout in Europe with any population only containing a limited genetic variation present in the species (Ferguson, 1989). The presence of genetic variability within the species, at a population and intraspecific level, has been essential for successful survival and reproduction in response to both short-and long-term environmental changes (Laikre, 1999).

There have been many studies, which aimed to determine genetic divergence associated with reproductive isolation within brown trout populations. Ryman et al. (1978) demonstrated evidence of two genetically distinct populations coexisting (Ferguson and Mason, 1981). Phenotypic differences (body size) coupled with significant allele frequencies provided evidence of the two distinct populations within the lake. The mechanisms involved, which led to genetic isolation, are unclear, however micro geographic separation has been suggested (Youngson et al., 2003). Molecular marker studies involving allozymes, mitochondrial DNA and microsatellites demonstrated that ferox brown trout in Lochs Awe and Laggan (Scotland) were reproductively isolated and genetically distinct from co-occurring brown trout. Although some introgression with sympatric brown trout was shown, ferox trout within the two lochs were shown to be a distinct biological, phylogenetic and morphological species (Duguid et al., 2006).

Lough Melvin has one of the few populations of trout left in Europe, remaining sympatric. Three types of brown trout are found there, known as the gilllaroo, sonaghen and ferox. They are distinct morphologically and have different

feeding preferences (Ferguson, 1986; Cawdery and Ferguson, 1988; Ferguson, 2004). Allozyme studies have shown major differences in the occurrence and frequency of alleles, indicating the three types are reproductively isolated. Further investigations of mtDNA restriction fragment length polymorphisms (McVeigh et al., 1995; Hynes et al., 1996), multi-locus DNA fingerprints (Prodohl et al., 1992), has proven the genetic integrity of the three types. The genetic discreteness has been maintained by geographical separation of the spawning sites (Ferguson and Taggart, 1991). The Gillaroo spawn in the only out-flowing river of the lake, while sonaghen spawn in the inflowing rivers. Ferox spawn lower down in inflowing rivers used by sonaghen, but spawn earlier in the autumn (Ferguson, 2004).

Brown trout populations inhabiting fluctuating environmental conditions are likely to possess specific adaptations. Many of these adaptations are manifested through unique life-history traits, such as time of spawning, spawning site, migratory pattern, feeding, age of maturity and growth rate (Ferguson, 2004). Also, anadromous behaviour has been shown to be highly heritable while influenced by environmental changes in temperature and water velocity (Euzenat et al., 1999; Jonsson and Jonsson, 2002). There is a difficulty in estimating the contribution of either factor in the control of anadromous behaviour (Jonsson, 1982; Nordeng, 1983; Palm and Ryman, 1999; Charles, 2005).

The levels of genetic differentiation between anadromous and non-anadromous brown trout coexisting in the Oir river (Normandy, France) was examined using fifteen microsatellite markers. Despite large amounts of variation, no genetic difference was found between the two morphs (Charles et al., 2005). Genetic studies on both morphs, anadromous and non-anadromous, have given some conflicting conclusions ranging from lack of genetic divergence (Hindar et al., 1991;

Cross et al., 1992; Pettersson et al., 2001) to some degree of isolation (Krieg and Guyomard, 1985; Krueger and May 1987; Skaala and Naevdal, 1989; Charles et al., 2005). Therefore, the distinct levels of genetic differentiation within brown trout populations must have functional and/or adaptive significance, which ensure the survival of the species irrespective of the life history adopted.

1.3 Recreational fisheries within the United Kingdom

Recreational angling has a long and distinguished tradition, with brown trout providing an irresistible quarry to many sport anglers. Specifically, life history traits have developed a diverse diet and the ability to feed within all levels of the water column. This has proved their undoing, as the tendency to rise to a well-presented artificial dry fly has captured the imagination of anglers for generations. However, due to anthropogenic changes in lotic chemistry and reduced local recruitment, many riverine systems lack the wild indigenous brown trout populations to sustain fishing and harvest pressures. With an estimated 430,000 trout anglers in England and Wales (EA, 2006), many stocks have been unable to cope with such angling pressures and therefore artificial stocking became necessary. Approximately 750,000 “catchable” farmed brown trout equating to 344 tonnes are stocked into the rivers/stillwaters of England and Wales each year (Spurgeon et al., 2001; Chatterji et al., 2007). Farm-reared fish for restocking have usually been cultured using fast growing commercial strains; however translocation of stocks has also been common. Elliott (1994) suggested that farmed brown trout have been stocked into rivers for at least the last 120 years.

The first brown trout farms were set up in 1868 by Armistead in Cumbria, and then in Solway (Dumfries, Scotland) and by Sir James Maitland at Howietoun in

1881 (Stirling, Scotland; Ferguson, 2006). Broodstock from both farms were based on the Loch Leven stock (Kinross, Scotland), however, broodstock from other locations and populations have been incorporated. Wild sea-trout strains were taken from the River Teith, a tributary of the River Forth and have been used at Howietoun to supplement broodfish and to provide for restocking purposes (Ferguson, 2006). Many of the brown trout farm stocks are derived originally from these Solway and Howietoun strains and therefore the majority of the farmed brown trout in the United Kingdom will be similar genetically (Ferguson, 2006). Therefore, there are growing concerns that the stocking of farmed fish affects the integrity of wild brown trout populations and that stocking sterile all-females these impacts may be minimised.

1.4 Genetic impacts of stocking on indigenous wild trout populations.

Genetic studies on fish populations have aimed to quantify the rates of mixing between farm origin and wild stocks as a result of stocking practices within freshwater fisheries (Ruzzante et al., 2004). Other studies have combined historical (scale) and contemporary samples that allowed allelic comparisons between populations before and after stocking (Hansen, 2002). This has allowed the quantification of genetic introgression (genetic mixing) within populations. The potential effects of genetic introgression from farm brown trout with wild brown conspecifics are a reduced fitness, loss of local adaptation and transfer of disease (DeWald and Wilzbach, 1992). Genetic introgression can be quantified according to the amount detected and range from no introgression detected (Moran et al., 1991) to moderate introgression (Taggart and Ferguson, 1986) to complete extinction of an original population (Hansen et al., 2001).

Farm-derived introgression has been shown to be much lower than expected in many studies taking into account the timescale of the stocking practices involved. Hansen et al. (1995) suggested that anthropogenic changes have led to declines in anadromous brown trout in the River Karup (Denmark). Re-stocking with large numbers of hatchery trout had occurred during the 1980s and the genetic contribution of hatchery trout was much less than would have been expected. Likewise, brown trout population structure was assessed in three recruitment streams in which stocking practices with exogenous brown trout had occurred for four decades (Heggens et al., 2005). Microsatellite markers indicated that only a small proportion of the fish sampled from the lake originated from the introduced stain (>3%). Hansen (2002) combined historical (scale) and contemporary samples (1986-2000) and indicated that the introgression from farmed-reared trout was approximately 6 % genetic contribution to the population, when the expected genetic contribution by farmed trout was 64 % of the population. This was assessed from the numbers of stocked trout and assuming equal survival and reproductive performance of wild and domestic trout. On the other hand, the reproductive performance of domestic brown trout can be increased by continued stocking of hatchery trout and therefore mixing of farm genes can occur where previously no introgression had been detected. Izquierdo et al. (2006) observed introgression of farm alleles in Spanish brown trout populations where previously, Moran et al. (1991) found only pure native individuals even though stocking had been undertaken prior to the study. This suggests that although stocked fish have been shown to perform poorer than wild fish once released, continued stocking may increase genetic introgression between wild and stocked populations.

Similarly, Araguas et al. (2004) studied the effects of stocking on wild brown trout populations in the Pyrenees. Stocking had resulted in introgressed populations where those with higher introgression coefficients also have the highest value of heterozygosity. The increase of local diversity reflects the decrease of genetic differentiation between populations and loss of native alleles. Halbisen and Wilson (2009) suggested stocking history alone was not indicative of admixture in these populations. It was found that half of the supplemented populations of lake trout (*Salvelinus namaycush*) included in their study exhibited genetic profiles consistent with native ancestry indicating limited introgression. The remaining supplemented populations, however, showed evidence of introgression and homogenisation with genetically distinct stocked fish. This suggests genetic or ecological factors facilitated reproduction between native and stocked fish.

Many studies, using a range of genetic techniques, have investigated introgression between wild and stocked brown trout. In French stocks within the Pyrenees, Berrebi et al. (2000) investigated the major impact of restocking, with introduced Atlantic strain (AT) brown trout on endemic Mediterranean (ME) lineage. The Mediterranean (ME) strain are characterised by the alleles LDH5*(100), TF (102) and FBPI*(150), whereas the Atlantic strain (AT) have LDH5*(90), TF*(100) and FBPI (100) as fixed alleles. The alleles have a high discrimination factor as the majority of the domestic strains belong to the Atlantic strain (AT), therefore it is possible to evaluate the domestic gene flow in ME populations. Sampling at 13 different stations in that region demonstrated that introgression by farm genes varied from 0 to 77 %. Also, it was suggested that if stocking practices continued on a European level, endemic strains would be replaced by uniform commercial domestic stocks, resulting in reduction in the species' genetic diversity. Bennet and Kershner

(2009) investigated introgression between native Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and introduced rainbow trout (*Oncorhynchus mykiss*) in the upper Kootenay river (British Columbia, Canada). The aim was to determine the genetic structure of the cutthroat population after 10 years of stocking diploid rainbow trout. After this stocking period a new programme was introduced whereby only triploids were stocked. The research used nuclear markers at 14 sites of sampling and indicated that introgression levels were 20-30 %, significantly increased at two of the three sites surveyed, suggesting the new stocking programme (triploids) did not appear to have reduced introgression or prevented the spread of hybrid individuals. Similarly, Araguas et al. (2008) sampled for genetic variation within ten populations (*S. trutta*) in the Pyrenees Mountains (Spain). Since 1997, the populations within this region were designated 'genetic refuges' prohibiting hatchery releases and under extensive fishing regulations. Genetic variation was measured in six genetic refuges (non-stocked) and in four non-refuges (stocked) within the same region to understand the effects of cessation of stocking. Each population was sampled four times over a thirteen year period (1993-2006). The analysis was based on the same allele which Taggart and Ferguson (1986) had investigated, LDH-C1*90, which distinguishes native from exogenous hatchery populations. Results indicated significant genetic introgression resulting from past stocking practices. However, new policies had prevented detectable introgression from increasing throughout the region. Hansen et al. (2009) analysed historical (scales from 1927-56) and contemporary samples to identify changes in the genetic composition of brown trout in six Danish rivers. Suspected changes in the genetic composition of the brown trout populations occurred through wild population declines and a stocking programme with non-native hatchery trout. It was shown that reduced effective

population size (N_e) of wild fish and continued stocking had increased introgression of hatchery fish into wild fish in the range of 14-64 %. It was argued that population declines had a limited negative effect, but introgression with hatchery trout may result in reduced local adaptation.

Many studies indicate high genetic introgression rates from introduced farm fish into wild trout populations. Sonstebo et al. (2008) studied the effects of stocking brown trout in three alpine lakes (Skavatn, Grondalsvatn and Nordmannslagen), using microsatellite data and indicated that high rates of introgression were observed within the lakes suggesting good survival and reproductive success of stocked fish. In addition, Berrebi et al. (2000) described the marble trout (*Salmo marmoratus*) as highly endangered due to hybridisation from intensive stocking. Confirmation of hybrids was achieved using 31 presumptive enzyme loci and showed the existence of extensive hybridisation in the main river resulting from alleles belonging to Marble, Danubian and Atlantic brown trout. Despite genetic introgression being high in the main Soca river channel, nearly pure populations of marble trout were found in the headwaters of five tributaries. Simmons et al. (2010) used single-nucleotide polymorphisms (SNPs) to detect introgression between subspecies of rainbow trout known as red-band trout, in the upper McCloud river (USA). Analysis suggested many areas contained a dramatic loss of populations free from introgression. Hansen et al. (2009) found strong introgression at a population level by hatchery strain trout as much as 70.8 %.

The rate of introgression is multifaceted within any given population and is predominantly affected by duration and intensity of stocking which increases homogenisation and admixture. Muhlfeld et al. (2009) found that hybridisation of wild cutthroat trout with stocked rainbow trout, is more likely to occur in streams

with warmer temperatures, increased land disturbances and proximity to the source of hybridisation. Blankenship et al. (2009) suggested stocking hatchery fish to increase fish numbers and improved catch returns, may be offset against the negative effects of genetic introgression and small effective population size (N_e) in hatchery fish. Also, it may be difficult to accurately evaluate the effects of stocking on brown trout biodiversity in any system if the magnitude of the "normal" variation in genetic composition over time is unknown (Laikre, 1999).

Ruzzante et al. (2004) examined polymorphisms at seven microsatellite loci among sea trout, collected from three areas in Limfjord (Denmark). The proportion of sea trout of hatchery origin varied widely, reflecting past stocking policies. High proportions of genes of farm origin were found in east Limfjord region (39.3 %), where stocking with domestic stocks was practiced intensely at the time of sampling, and in the west (57.2 %) where a coastal stocking programme of post-smolts took place over several years in the early 1990s. In contrast, in the central Limfjord, where stocking with domestic trout was abandoned in the early 1990s, the proportion of sea trout of domestic origin was 8.5 %. For all three regional groups, virtually no sea trout of hatchery origin were found among spawning individuals. This suggests domestic origin anadromous brown trout experience significantly higher sea mortalities. Similarly, Hansen et al. (2000) conducted a stocking impact assessment of brown trout using microsatellite DNA markers in Denmark. Results indicated 46 % farm trout introgression in the freshwater component compared to <7 % in the sea trout component. In addition, comparison of nuclear genes and mtDNA indicated that there was a greater influence of male farm trout suggesting males had a greater tendency to remain in freshwater.

Genetic introgression can be enhanced by a decrease of natural reproduction of wild trout stocks (*S. trutta fario and S. marmoratus*) within the Adriatic and Danubian basins of Switzerland. Protein loci data indicate the replacement of native stocks of the Adriatic with introduced Atlantic (AT) based origin. The high introgression rates seem consistent when accounting for the stocking activities within the drainages. Also, introgression is enhanced by a decrease of natural reproduction caused by deterioration of trout habitats through anthropogenic activities (Largiader and Scholl, 1995).

The potential for introgression caused by semi-supportive breeding (using locally sourced strains as broodstock) has been less documented. In Norway, Wollebaek et al. (2010) assessed potential introgression of a brown trout population in an alpine lake. Assessment involved 13 polymorphic microsatellite loci and found natural straying and non-native reproduction especially among wild populations inhabiting environmentally unstable habitats. However, the retention of genetic structure across spawning tributaries indicated low reproductive success of wild-born non-natives. Genetic structure among tributaries had not been influenced by semi supportive breeding, because of recruitment failure of stocked fish. Other factors, such as physical barriers have been shown to reduce introgression of farmed genes into wild populations. Van Houdt et al. (2005) analysed the contribution of farm genes into wild Belgian brown trout and found very limited or total absence of farm genes upstream of physical barriers. Brown trout migration is reduced in the presence of obstacles, preserving the genetic integrity of the populations upstream of the barrier. Therefore, changes to stocking policies may reduce introgression and preserve the genetic integrity of remaining wild brown trout populations.

1.5 Fisheries legislation and policy within the United Kingdom

The extensive genetic studies described reveal an abundance of genetic heterogeneity within the taxa *Salmo trutta L.* Within the five evolutionary lineages there are distinct differences within populations, as demonstrated by Lochs Melvin, Awe and Laggan, which exhibit pronounced differences among local populations (Ferguson, 2004; Duguid et al., 2006). As a result an increased amount of genetic variation is represented by differences between populations. Brown trout exhibit many phenotypes varying in morphology, ecology, life history stages and behaviour (Ferguson, 2004). In addition, the ongoing stocking with farmed fish into freshwater fisheries and the harvest of wild trout within sport fisheries has threatened wild brown trout population structure and genetic integrity (Laikre, 1999). Therefore, conservation of the species must begin at the local population level to conserve the remaining genetic integrity of wild brown trout populations.

Supportive stocking using locally sourced broodfish and stocking with sterile trout have now become part of watershed management plans (EA, 2009). This is advocated as an alternative to stocking domesticated trout in many countries. For example, domesticated trout had genetically affected brown trout populations in Denmark, whereas stocking wild exogenous trout into one of the rivers had little or no impact (Fritzner et al., 2001). Improvements in water quality in German rivers have allowed for recovery of some brown trout populations. However, whether improvements were sufficient to maintain stocks and angling yield without stocking was investigated by Baer and Brinker (2010). Natural reproduction was recorded on the River Wutach and a stable stock of trout >20 cm was found. Angling yield and perception associated with the halt of stocking was recorded and indicated that 60 %

of anglers were convinced stocking was unnecessary and trout catches increased after stocking ceased.

The Environment Agency (EA) has introduced legislation to protect wild brown trout in England and Wales. Under the umbrella of the National Trout and Grayling Fisheries Strategy (TGFSG), brown trout stocked into all but totally enclosed waters with no significant natural brown trout populations will be with either sterile all-female triploid brown trout or brown trout from breeding programmes using locally sourced broodfish (EA, 2009). This decision has followed an extensive consultation process evaluating its contribution towards the good ecological management of freshwater bodies forecast for 2015, as defined in the EU Directive 2000/60/CE of 23/10/2000. Three steps were taken prior to implementing the legislation, which included: bibliography survey, risk analysis of stocking with triploids and common-garden experiments. The studies examined survival, growth and catchability of all-female triploid with respect to wild or farmed diploids. Results indicated that all-female farmed triploids: 1) had a similar catchability than to all-female farmed diploid, 2) the stocking of all-female triploids did not noticeably impact on wild stocks, 3) all-female triploids had the same feeding regime as farmed and wild diploids, 4) all-female triploids did not exhibit reproductive migration to spawning grounds, and 5) had a higher rate of capture at the end of the fishing season (Piferrer et al., 2009). Therefore, after a public consultation it was concluded that all-female triploids could be used for freshwater sport fisheries. The implementation plan was conducted over a 5 year period with the overall objective to reduce the use of farmed diploids by 30 % in 2010 and by 50 % in 2013 until their replacement with all-female triploids in 2015 (Piferrer et al., 2009). Genetically sterile all-female

triploids will prevent genetic introgression through sterility thus contributing to good ecological management of freshwaters (EA, 2010).

The techniques used to produce triploid fish are chromosome set manipulation and therefore the Department for the Environment Food and Rural Affairs (Defra) and the European Inland Fisheries Advisory Commission (EIFAC) does not define triploids as Genetically Modified Organisms (GMOs). According to current regulations in the European Union, fish produced by polyploidy induction are not considered GMO's under the relevant directive (Directive 2001/18/EC European Parliament-Article 2-Annex 1A-Part 2).

2. Polyploidy utilisation in aquaculture

2.1 Triploidy induction

The induction of triploidy is a technology used to induce sterility in animals, which like the sterilisation of terrestrial animals by castration (bulls, pigs, and poultry) can increase productivity and improve meat quality (Piferrer et al., 2009). Triploidy is induced by a physiological shock (temperature, pressure or chemical) applied to newly fertilised eggs causing the retention of the second polar body. In fish, eggs are released at the metaphase stage of meiosis II with further development of the egg triggered by fertilisation causing the resumption of meiosis II (Figure 1). The application of a physical or chemical shock at this stage of development can prevent second polar body extrusion while allowing chromosomal division (Piferrer et al., 2009). Polar bodies have been shown to be a by-product of meiotic division, which degenerate shortly after formation allowing cytoplasmic volume to be maximised during meiosis II (Schmerler and Wessel, 2011). Triploid fish have been produced by pressure shock (Thorgaard et al., 1981; Chourrout, 1984; O' Flynn 1997; Benfey et

al., 1988; Cotter et al., 2000; Benfey, 2001; Friars et al., 2001; Schafhauser-Smith and Benfey, 2001; Brydges and Benfey, 1991; Hussain et al., 1991; Oppedal et al., 2003; Johnson et al., 2004; BTA 2006; Peruzzi et al., 2007; Garner et al., 2008; Chiasson et al., 2009), heat shock (Sutterlin et al., 1987; Benfey, 1999; Gillet et al., 2001; Rougeot et al., 2003,); and cold shock (Piferrer et al., 2003; Dias da Silva et al., 2007) or chemical shocks using cytochalasin B in shellfish (Gendreau and Grizel 1990; Hand and Nell, 1999). In addition crossing diploid and tetraploid individuals can produce triploids. Table 1 gives key studies of triploid induction in finfish.

In order to obtain 100 % triploidy and high survival rate, a precise species-specific protocol is required (Piferrer et al., 2009). Currently there are reliable triploid induction protocols for a number of key salmonid species important to aquaculture and fisheries. The fundamental variables of triploid induction, which influence the effectiveness of shock, are the timing, intensity and duration of the shock (Felip et al., 1997; Piferrer et al., 2009). Pressure shocks consist of a sharp increase in hydrostatic pressure applied to newly fertilised eggs. The underpinning mechanisms of pressure induction are still not fully understood; however it may involve pressure acting on the egg to resist polar body extrusion, or disrupting meiotic spindle formation (Dasgupta, 1962; Zhu et al., 2007). It has been shown there is a “window” where triploidy can be induced and optimised, while the application of the shock outside this window result in reduced triploid rate and survival (Hussain et al., 1995). Timing of the shock is intrinsically linked to temperature and the rate of embryonic development, therefore the optimum shock is dependent on the induction temperature used to fertilise gametes.

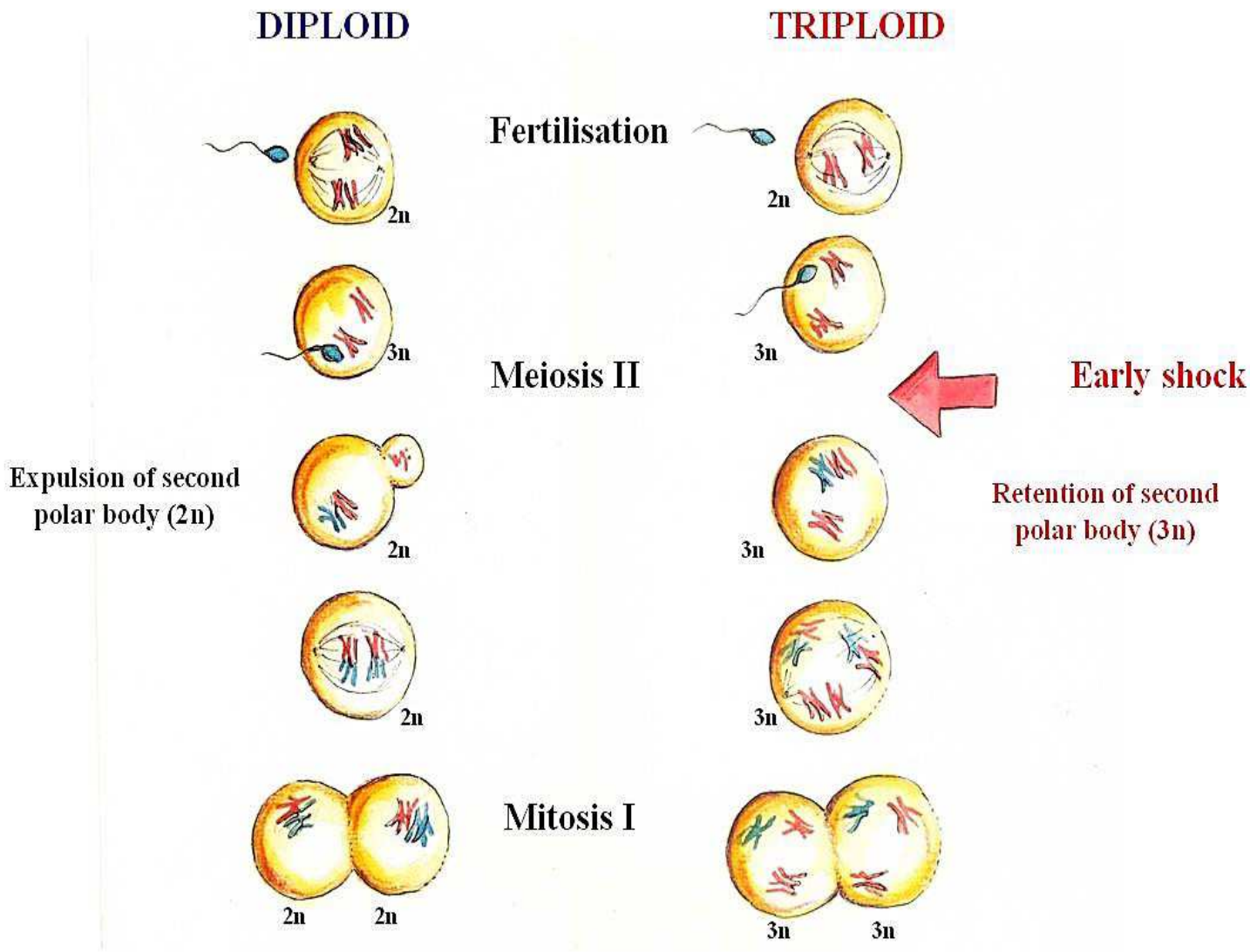

Figure 1. Ploidy manipulation in fish. Eggs are released at metaphase of meiosis II. Fertilisation resumes meiosis. A physical or chemical shock applied during meiosis can suppress cell division while allowing chromosomal division, producing triploids (meiosis suppressed) by retention of the second polar body (Adapted from Dr. Tom Hansen, IMR, Norway).

The use of tau zero (T_0), a unit of relative embryonic development equivalent to one mitotic cycle during synchronous cell division has been proposed by Gomelsky (2003) in order to standardise shock timing. Once the species-specific duration of one mitotic cycle is known at a particular temperature, early developmental stages where shock application is effective can be defined in either developmental degree minutes or T_0 (Piferrer et al., 2009). However, other studies have used degree-minutes of development as an indicator of precision, which is the current technique employed commercially for Atlantic salmon (*Salmo salar*) production.

The duration and intensity of the shock must be sufficient to cause polar body retention without causing excessive physiological stress to the fertilised gametes. Hussain et al. (1991) found lower pressure intensities (7,000-7,500 psi) failed to induce triploidy in Nile tilapia, *Oreochromis niloticus*, while higher intensities reduced survival. Also, Quillet et al. (1995) tested heat shocks of 25-29 ºC applied for 10-20 minutes to optimise triploidy in brown trout and found that an intensity of 26-27 ºC was optimal with the highest induction and survival rates.

Table 1. Key studies showing triploid induction treatment in commercially important species for aquaculture and fisheries.

Species	Triploidy treatment	Authors
Atlantic Salmon *Salmo salar*	Hydrostatic pressure/Heat	Benfey and Sutterlin (1984)
	Hydrostatic pressure	Friars et al. (2001)
	Heat	Sutterlin et al. (1987)
Chinook salmon *Oncorhynchus tshawytscha*	Hydrostatic pressure/Heat	Johnson et al. (2004)
Caspian salmon *Salmo trutta caspius*	Heat	Kalbassi et al. (2009)
Coho salmon *Oncorhynchus kisutch*	Hydrostatic pressure/Heat	Teskeredzic et al. (1993)
	Hydrostatic pressure	Piferrer et al. (1994)
Chum salmon *Oncorhynchus keta*	Hydrostatic pressure	Benfey et al. (1988)
Brown trout *Salmo trutta*	Heat	Arai and Wilkins (1987)
	Heat	Altimiras et al. (2002)
	Hydrostatic pressure	BTA (2006)
Lake trout *Salvelinus namaycush*	Hydrostatic pressure	Kozfkay et al. (2005)
Rainbow trout *Oncorhynchus mykiss*	Hydrostatic pressure	Chourrout (1984)
Salmo gairdneri	Hydrostatic pressure	Lou and Purdom (1984)
Salmo gairdneri	Heat	Thorgaard et al. (1982)
Salmo gairdneri	Heat	Solar et al. (1984)
Brook trout *Salvelinus fontinalis*	Hydrostatic pressure	Benfey and Biron (2000)
	Hydrostatic pressure	Schafhauser-Smith and Benfey (2001)
Arctic charr *Salvelinus alpinus*	Hydrostatic pressure	Gillet et al. (2001)
Turbot *Scophthalmus maximus*	Cold	Cal et al. (2005)
	Cold	Piferrer et al. (2000; 2003)
Yellowtail flounder *Limanda ferruginea*	Hydrostatic pressure	Manning et al. (2004)
European Sea bass *Dicentrarchus labrax*	Hydrostatic pressure/Cold	Peruzzi and Chatain (2000)
	Hydrostatic pressure	Peruzzi et al. (2005)
	Cold	Felip et al. (1997; 2001)
Gilthead seabream *Sparus aurata*	Cold	Haffray et al. (2005)
Yellow croacker *Pseudosciaena crocea*	Hydrostatic pressure	Xu et al. (2008)
Eurasian perch *Perca fuviatili*	Heat	Rougeot et al. (2003)
Yellow perch *Perca flavescens*	Hydrostatic pressure/Heat	Malison et al. (1993)
Grass carp *Ctenopharyngodon idella*	Hydrostatic pressure/Heat	Cassani and Caton (1986)
Common carp *Cyprinus carpio* L.	Hydrostatic pressure/Cold	Linhart et al. (2001)
Mozambique tilapia *Oreochromis mossambicus*	Heat	Varadaraj and Pandian (1990)
	Pressure/heat	Hussain et al. (1991)

The induction method used to induce triploidy has been shown to affect survival. For example, temperature shock on newly fertilised eggs resulted in reduced survivals in Caspian salmon (*Salmo trutta caspius)* (Kalbassi et al., 2009). In addition, the highest triploid yield obtained in this study was 57 %, indicating the need for an optimising induction protocol to deliver acceptable triploid yield and survival. Importantly, low triploid yields are unsustainable both economically but also environmentally. The selection of a given triploid induction method (pressure, temperature or chemical shock) depends largely on the species, the availability of a given technique or protocol (e.g. pressure vessel) and previous knowledge available. In rainbow trout, both pressure and thermal treatments were shown to give similar triploid rates (97.5 % ± 2.5 and 92.9 % ± 5.6, respectively); however, survival was significantly higher after pressure rather than thermal treatment (Haffray et al., 2007). It has become the consensus that hydrostatic pressure shock is the methodology of choice for triploid induction in salmonids due to the relative ease to standardise the shock, as compared to temperature, and to obtain consistent results while reducing the physiological burden associated with temperature shocks (Piferrer et al., 2009).

2.2 Importance of fine-tuned variables and egg quality to induce triploidy

As with diploid aquaculture production, the quality of the gametes obtained for the induction process has a major influence on the survival of triploids once the shock parameters have been accurately defined. Under either wild or captive conditions, gamete quality can be highly variable and be influenced by a number of environmental factors or broodstock management practices (Bode and Labbe, 2010). Many factors such as time of stripping, degree of over-ripening, and broodstock

rearing temperature have significant effects on gamete quality and progeny survival (Bromage et al., 1992). Other factors, such as diet can influence egg quality in rainbow trout (Satoh et al., 1986), and time of stripping has been shown to influence survival to the eyed stage in rainbow and brown trout (Springate et al., 1984; Samarin et al., 2008; Bahrekazemi et al., 2009). Other biochemical factors such as ovarian fluid pH influences sperm motility in rainbow trout (Wojtczak et al., 2007) and inorganic ions (Ca+) were associated with increasing motility and fertilisation success in Chinook salmon (*Oncorhynchus tshawytscha Walbaum 1792*; Rosengrave et al., 2009).

The key egg quality indicators used are visual inspection of morphology (colour and texture) and fertilisation, and hatching rates (Lahnsteiner, 1999, Manour et al., 2007). As triploid induction exerts additional stress on the egg, it is important that egg quality is maximised or resultant lower survival during incubation will be seen as demonstrated in previous studies (McGeachy et al., 1995; O'Flynn et al., 1997; Benfey, 2001; Cotter et al., 2002). In addition, Taylor et al. (2011) suggested egg quality had a greater effect on survival than did ploidy in Atlantic salmon. Subsequently, to maximise triploid induction and survival rates the induction process should be optimised in terms of type of shock used, duration and intensity. In addition, egg quality appears to be a fundamental component determining survival post-triploid induction. Over-ripening can lead to spontaneous triploidy, therefore, a combination of if use over ripe eggs then HP shock, can lead to decreased survival, increased mortality rate and failure to induce triploidy (Aegerter & Jalabert, 2004).

2.3 Ploidy level determination

The experimental or commercial induction of triploids must be followed by an accurate determination of the expected ploidy levels in the manipulated animals (Piferrer et al., 2009). Triploids possess three sets of chromosomes due to polar body retention and therefore have larger nuclei to facilitate the increase of genomic content. In this respect a low cost and relatively simple indirect method involves the measurement of the major and minor axis lengths of erythrocytes (Benfey, 1999). Other methods to identify triploid fish include chromosome preparations (Pradeep et al., 2011), Coulter counter (Johnson et al., 1984), flow cytometry (Peruzzi et al., 2005) and microsatellite markers (Hernández-Urcera et al., 2012). However, methods, which can screen large numbers of fish such as the direct method of flow cytomery, are more applicable commercially (Lecommandeur et al., 1994). Flow cytometry (FC) analysis is very sensitive and allows large number of cells to be analysed (between 5,000 and 15,000 per analysis) per sample. Using FC analysis samples can be kept frozen for extended periods, smaller amounts of sample material are needed for the analysis and several hundred samples can be analysed every day (Thorgaard et al., 1982). However, aforementioned techniques have advantages and limitations due to costly apparatus, destructive nature of sampling and/or the speed of the procedure not being suitable for screening large numbers of experimental or commercial groups. On the other hand, an economic and robust method for the determination of ploidy status in brown trout would be advantageous to the aquaculture and freshwater fisheries to ensure compliance with legislation introduced by the Environment Agency. The screening of stocked trout will take place by an auditing process and therefore 100 % triploid rate is required prior to stocking permits being granted within England and Wales. Therefore, an early ploidy

verification technique applicable at any postembryonic stage of development may decrease the need to on-grow commercial trout to a size where blood or tissue can be collected.

2.4 Triploid growth performance

The growth of triploid fish has attracted the greatest amount of attention, as this could mean increased productivity and profitability for farmers. Piferrer et al. (2009) suggested that triploid fish might theoretically grow faster than diploids, by virtue of (1) increased cell size or "the gigantism effect", (2) the possession of greater genomic heterozygosity and (3) the diversion of energy from gonadal development into somatic growth. However, a physiological shock shortly after fertilisation can cause stress on the developing embryo if an optimised protocol is not used. Therefore, many studies in salmonids indicate triploidy does not result in any significant increases in growth rates over diploids in the juvenile stages of Arctic charr (*Salvelinus alpinus*) (Chiasson et al., 2009), rainbow trout (Wagner et al., 2006) and African catfish (*Clarius gariepinus*) (Karami et al., 2009). However, in the freshwater phase and post-seawater transfer triploid Atlantic salmon outgrew their diploid counterparts (Taylor et al., 2011, 2012). In addition, adult triploid brook trout (*Salvelinus fontinalis*) were shown to have increased body length (i.e. lower condition factor) in comparison to diploids after three years of growth (Schafhauser-Smith and Benfey, 2001). This increase in growth is apparent during the spawning season, when diploids reduce somatic growth in favour of gonad development while triploids continue to allocate energy reserves to somatic growth. Similarly, triploid turbot (*Scophthalmus maximus*) exhibit similar growth to diploids pre-maturation and improved growth and survival post-maturation (Cal et al., 2006).

Many studies indicate only small differences between ploidy in growth rates, with triploids growing comparably or slightly slower than diploids when communally reared (Quillet and Gaignon, 1991; Galbreath et al., 1994; McGeachy et al., 1995; Withler et al., 1995; McCarthy et al., 1996; Johnson et al., 2004; Mori et al., 2006). However, growth rate was not different between diploid, triploid, and mixed groups of diploid/triploid Chinook salmon subject to growth performance trials in freshwater tanks (Garner et al., 2008). This was in contrast to a recent study by Taylor et al. (2014), where growth in seawater is severely compromised in triploids under communal ploidy rearing, yet Fraser et al. (2013) indicated that no differences of growth in seawater cages. Conflicting results in growth performances of triploid were reported during the saltwater on-growing phase with some studies indicating that triploid Atlantic salmon out-perform diploids (O'Flynn et al., 1997) or diploid chinook salmon out-perform triploids (Shrimpton et al., 2011) but triploids can suffer greater mortalities. Galbreath and Thorgaard (1995) found that survival to harvest of triploids and diploid reared in sea cages was 40 and 65 % respectively. Higher mortalities were observed in triploids on seawater transfer probably due to incomplete smoltification and a general trend towards reduced growth and survival in comparison with diploids. Taylor et al. (2011) indicated that triploid Atlantic salmon (S0+) smolted earlier than diploids using a square wave photoperiod indicating other factors such as differential smoltification were the cause of increased mortalities seen in earlier studies (Boeuf et al., 1994). In addition, triploid rainbow trout and Atlantic salmon exhibit greater muscle fibre and muscle fibre hypertrophy than diploids (Suresh and Sheehan, 1998; Sheenan et al., 1999; Poontawee et al., 2007). Bonnet et al. (1999) observed growth performance in diploid and triploid brown trout and found that diploids were of significantly greater body weight than triploids during

grow-out trials in seawater. Therefore, the majority of studies indicate triploids do not grow quicker than diploids in the juvenile stages of development. However, it is possible for triploids to outgrow diploids during the spawning season when diploids reduce somatic growth in favour of gonad development.

2.5 Physiology of triploid fish

The functional consequences of triploidy are increased nuclear size and a reduction in cell numbers. Benfey (1999) reviewed such findings and indicated increasing cell sizes led to decreased cell numbers in different fish organs, such as brain, retina, epithelia, cartilage, muscle, liver, kidney, testes, ovaries and blood count, concluding that the increase in cell size did not appear to confer any growth advantage to triploids due to the concomitant decrease in cell numbers. It is also unknown whether (i) increased cellular volume may lengthen internal distances, and compromise signal transduction pathways and (ii) the reduction of surface to volume ratio resulting from increased cell volume impairs transport processes across membranes (Maxime, 2008). Many studies show triploids are physiologically similar to diploids with only subtle differences between ploidy. However even in the absence of larger physiological differences between ploidy changes in cell size may affect the rates of respiratory gas exchange across the cell membrane (Atkins and Benfey, 2008).

Triploid salmonids have been reported to be more sensitive to environmental changes than diploid fish, which may relate to their altered physiology and cellular morphology. Ching et al. (2010) suggested diploid and triploid Chinook salmon are phenotypically indistinguishable, except under stress where triploids show reduced performance. In particular, sudden increases in water temperature during culture results in increases in temperature-related mortalities in triploids (Aldrin, 1984;

Dubuisson, 1987; Beauvineau; 1988; Ojolick et al., 1995; Altimiras et al., 2002). This suggests an altered tolerance of triploids to physico-chemical factors such as oxygen, temperature and salinity changes. However, although there are haematological differences described between diploid and triploid fish (Benfey, 1999), little difference has been observed in the aerobic capacity and cardio-respiratory physiology of triploids specifically in trout and salmon (Maxime, 2008).

Critical swimming speed has been used as an indicator of aerobic capacity in fish and it was shown triploidy did not affect swimming performance in brown trout (Altimiras et al., 2002). Additionally, cardiac performance and blood circulation appear unaffected by triploid status in brown trout (Mercier et al., 2002). This has relevance to triploid brown trout stocking and indicates fighting qualities and stocking location may not be inhibited by triploid status. Concerns regarding stocking triploid brown trout into fast flowing rivers should not be warranted, as swimming capabilities seem unaffected by triploid status. However, jaw and skeletal malformations are known to be more prevalent in triploids, and these are known to effect swimming capabilities in diploids (Hansen et al., 2010). Triploid brook trout and rainbow trout do not differ from diploids in their critical thermal maxima (CTM), although it was noted that females exhibit higher CTMs than males (Benfey et al., 1997; Galbreath et al., 2006). The characteristic larger cell size of triploids does not limit physiology or recovery from exercise or maximum swimming speeds in brook trout (Cotterell and Wardle 2002; Hyndman et al., 2003a). However, Hyndman et al. (2003b) found that triploid status did affect thermal tolerance of triploid brook trout. At chronic high temperatures (19ºC) triploids had difficulty in utilising anaerobic pathways and took longer to recover from metabolic disturbances. Lack of recovery was suggested as the key factor to increased mortality of triploids.

The majority of triploids (90 %) had died within 4 hours exposure to 19 ºC, whereas no diploids died. This may have significant consequences for restocking fisheries, specifically when summer temperatures are highest and remain high for long periods.

In addition, higher metabolic rates have been shown in triploids at lower temperatures, and lower metabolic rates at higher temperatures when compared with diploid siblings, demonstrating that triploids have a lower thermal tolerance than diploids (Ojolick et al., 1995; Mercier et al., 2000; Altimiras et al., 2002; Mercier et al., 2002; Hyndman et al., 2003b; Atkins & Benfey, 2008). Increased mortality in triploid rainbow trout has been reported when the temperature is at its highest in seawater (Ojolick et al., 1995). At higher temperatures when oxygen saturation is lower the reduced oxygen carrying capacity of triploids may be a factor explaining the increase in mortality at higher temperatures (Stillwell and Benfey, 1996). This may be significant in fisheries where triploids are stocked and "catch and release" is practiced. The ability of triploids to fully recover from sustained exercise through capture may be compromised specifically during warmer temperatures; however this remains to be proven. Lijalad and Powell (2009) indicated that triploid recovery from excess post-exercise oxygen consumption (EPOC) was quicker than diploids, having not incurred the same oxygen debt or the ability to repay oxygen debt quicker. With lower total blood haemoglobin and erythrocyte haemoglobin than diploids, it was suggested the aerobic capacity of triploids could be reduced. Similarly, no difference was observed in critical swimming velocity or consumption rates of carbon dioxide and oxygen between diploid and triploid Chinook salmon (Bernier et al., 2004). In addition, ploidy did not impair carbon dioxide transport or acid-base balance in Chinook salmon during sustained exercise. This may infer gas exchange across the

cell membrane is not a limiting factor in triploids as previously suggested (Atkins and Benfey, 2008).

Triploid brook trout, rainbow trout and Atlantic salmon also display a similar acute stress response as observed in diploid fish (Biron and Benfey, 1994; Benfey and Biron, 2000), suggesting that mortalities reported under commercial conditions cannot be attributed to differences in their physiological response to stress during husbandry or management practices (Sadler et al., 2000b). In addition, stress induced by transportation and handling was investigated by Wagner et al. (2006) using three strains of triploid and diploid rainbow trout with no significant difference in mortality observed between ploidy status exposed to changes in pH and temperature post-transportation. In other species, such as South American catfish (*Rhamdia quelen*), triploids have been shown to perform similarly to diploids when exposed to an environmental stressor (Weiss et al., 2010). Accumulated mortalities were similar between ploidies when exposed to continuous ammonia (NH_3) over a 96-hour period (Weiss & Zanibani-Filho, 2009). However, Virtanen et al. (1990) indicated swelling of the erythrocytes and accumulation of anaerobic metabolites in triploid rainbow trout with a decrease in swimming capacity. Increases in blood haematocrit and plasma lactate were found to be more pronounced in triploids indicating that the ability to expel metabolic wastes may be limited in triploid rainbow trout. Maxime, (2008) concluded that triploids in general are specifically affected by poor water quality in rearing ponds and are unable to cope with sources of chronic stress. In summary, according to the available data published on triploid fish, it appears that triploids could be more sensitive to sub-optimal environments, especially sudden increased in water temperature and decreased oxygen saturation. However, the aerobic capacity and blood circulation of triploid brown trout appear unaffected by

triploid status. In addition, studies showed similar acute stress response between diploids and triploids when exposed to handling or environmental stressors.

2.6 Haematological differences and immune function

The cellular status of triploid fish induces haematological changes, which have been investigated in a number of studies. Mean red blood cell volumes in Atlantic salmon were shown to differ between ploidy with 307 μm^3 in diploids compared with 432 μm^3 in triploids (Cogswell et al., 2002). In addition, increased mean corpuscular volume (MCV) results in a decrease of the surface-to-volume ratio of erythrocytes, mean volume and haemoglobin concentration but also lower erythrocyte numbers (Ranzani-Pariva et al., 1998). Differences observed in haemoglobin concentration will alter the carrying capacity of the blood and therefore aerobic capacity (Atkins and Benfey, 2008). In addition, Wang et al. (2010) noted triploid rainbow trout display similar nuclear erythrocyte morphology to atypical amphibian cells, with nuclear constrictions and abnormal divisions. The altered nuclear morphology may have an impact on haemoglobin carrying potential of those cells therefore reducing overall efficiency. Lower blood haemoglobin levels have been described in triploid Atlantic salmon (Benfey and Sutterlin, 1984), Coho salmon (Small and Randall, 1989), European sea bass, *Dicentrarchus labrax* (Peruzzi et al., 2005) and Caspian salmon (Dorafshan et al., 2008). However, it was suggested compensatory mechanisms account for alterations of haemological factors in triploid rainbow trout. Triploid Chinook salmon have lower oxygen consumption rates and lower arterial oxygen content than diploids (Bernier et al., 2004). In addition, triploids had a smaller oxygen carrying capacity than diploids, which may result in a reduced aerobic capacity during sustained exercise. Maxime and Labbe (2010) found that

ploidy-related differences in erythrocyte osmoregulation had no consequence at the whole organism level, and that triploid red blood cells have a better ability to face osmotic challenge. It has been suggested that the lower capacity of aerobic metabolism of triploids can lead to a depletion of energy stores through a reduced circulating number of erythrocytes (Ballarin et al., 2004). Therefore, the ability to repay the oxygen debt because of immunocyte activation may be limited. This could be related to the reduced total blood haemoglobin capacity of triploid erythrocytes, as shown in triploid Caspian salmon (Dorafshan et al., 2008).

Blood composition has been shown to be similar with lower leukocyte number for triploid rainbow trout (Svabodova et al., 1998) or higher percentages of granulocytes in triploid brook trout (Wlasow et al., 2004). In tench (*Tinca tinca),* no difference was observed in leukocyte counts between diploids and triploids at three years of age (Svabodova et al., 2001). Triploid turbot *Scophthalmus maximus* immune cells are typically bigger and fewer in number than diploids, with *in vitro* studies indicated similar respiratory burst activity and phagocytosis per microliter in diploid and triploid neutrophils (Budiño et al., 2006). Haematological indices are affected by triploidy in rainbow trout with triploids exhibiting reduced white blood cells (WBCs) following seawater transfer (Taylor et al., 2007). However, there was no difference in neutrophil activity between diploid and triploid rainbow trout showing that non-specific defence mechanisms are similar and that triploidy does not increase ploidy susceptibility to disease (Yamamoto and Lida, 1995; Dorafshan et al., 2008). Similar conclusions have been made in non-specific defence activity or response to immune challenge following vaccination in triploid and diploid fish (Langston et al., 2001; Svobodova et al., 2001). However, triploidy has been shown to affect the B-cell proportions in tissues and ploidy status affected the response of

both neutrophil and B-cell proportions to vaccination in S0 Atlantic salmon smolts (Fraser et al., 2012b).

Other studies also indicated no adverse effects of ploidy on disease and carcinogen resistance in salmonids. LaPatra et al. (1996) exposed diploid and triploid hybrid male brown trout and female lake trout (*Salvelinus namaycush*) to infectious haematopoietic necrosis virus. Triploid hybrids exhibited significantly better survival than diploid controls when exposed to waterborne virus (98 % triploid vs. 47 % diploid). Thorgaard et al. (1999) exposed diploid and triploid fry to three carcinogens (7, 12 dimethylbenz (*a*) anthracen, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and aflatoxin B1) and found that tumour occurrence was substantially lower in triploids than diploids in the swim bladder, stomach and kidney and moderately lower in the liver. Therefore, the haematological differences of triploids result in reduced surface to volume ratio of erythrocytes and lower numbers of white blood cells. However, lower WBCs may be compensated for by higher cell activity, as triploids have exhibited higher survival when exposed to waterborne virus. Studies indicate that triploids have lower haemoglobin levels than diploids, which may limit physiological response times and alter aerobic capacity during sustained exercise.

2.7 Deformity prevalence in triploid fish

The prevalence of deformities is a major constraint to the production of triploids in aquaculture (Taranger et al., 2010). Triploids demonstrate similar deformities to diploids however at a higher incidence than reported in diploids. Several studies indicate a higher occurrence of lower jaw deformities (LJD) in triploid salmonids compared with diploids in seawater (Sutterlin et al., 1987; Jungalwalla, 1991; McGeachy et al., 1995; Saddler et al., 2001). Other deformities include reduced gill

surface area (GSA) and gill filament deformity syndrome (GFD) (Sadler et al., 2001). Ocular cataracts are found to be consistently higher and more severe in triploids than in diploids (Wall and Richards, 1992). Leclercq et al. (2011) observed increased cataract prevalence and severity in two full-sib families of out-of-season triploid Atlantic salmon smolts compared with diploids. In other species, Grimmett et al. (2011) observed increased spinal deformities in triploid grass carp (*Ctenopharyngodon idella*) and suggested that triploid status increased spinal malformations in this species.

The incidence of deformities is an animal welfare concern and can reduce the commercial value and aesthetics of the fish resulting in production losses. The severity of vertebral abnormalities has been shown to effect growth performance. For example, the number of deformed vertebrae significantly influenced diploid Atlantic salmon growth with fish having higher numbers of deformed vertebrae also having lower weight than normal fish (Hansen et al., 2010). Similarly, many studies have reported increased prevalence of morphological deformities in triploid fish leading to lower survival rates during early development including salmonids (Cotter et al., 2002), sea bass (Zanuy et al., 1994), red sea bream (*Pagrus major*) (Sugama et al., 1992). Lijalad and Powell (2009) studied the physiological effect of lower jaw deformity (LJD) in all female diploid and triploid Atlantic salmon and found no effect of either ploidy or LJDs on critical swimming speed, but triploids with LJDs were not capable of attaining the same critical swimming speed after 45 min recovery. While studies indicate the consequences of increased deformity prevalence in triploid salmon, little is known regarding deformity prevalence in triploid brown trout.

Triploids cannot directly be improved by selection as they are sterile however they could be improved by selection of their diploid parental lines if traits measured in diploid are the same as triploids (Piferrer et al., 2009). In addition, certain stocks and or families may be more genetically predisposed to deformity, advocating the use of selection programmes for broodstock. Previous studies have investigated the genetic parameters in triploid fish and genetic correlations with diploids. This involves the production of families to estimate the variance component due to both environmental and genetic sources and their interactions therein (Piferrer et al., 2009). Several approaches have been use to compare the performance of diploid and triploid full-sib families (Johnson et al., 2004; Shrimpton et al., 2007; Chiasson et al., 2009). Generally these studies indicate significant family and ploidy interactions for traits such as growth, condition factor and sensitivity to photoperiods. Triploid vertebral deformities were found to be more prevalent in the fastest growing triploid families suggesting a genetic component to deformity prevalence. However, Blanc et al. (2005) reported that ploidy effects may not be true ploidy effects, instead a reflection of the variation in the maternal influence on performance traits due to the additional maternal genetic material (2^{nd} polar body retention). This indicates that during selective breeding more importance should be given to maternal selection than paternal selection (Fraser et al., 2012a).

Sub-optimal husbandry conditions such as temperature shocks during incubation have been shown to affect deformities in farmed fish. Ornsrud et al. (2004) investigated whether the combination of high vitamin A along with elevated water temperatures could cause deformities in salmon and that temperature was the main factor causing bone deformities (such as warped gill opercula, fin and jaw deformities). Indeed, heat shock during egg incubation can induce caudal vertebral

body deformities early in development of diploid Atlantic salmon (Wargelius et al., 2005). On the other hand, egg-rearing temperature has been shown to increases egg mortality and heart deformities in triploids (Fraser et al., 2013a).

The number of observed deformities observed in triploids seems to be related to the induction method used to produce them. Triploid trout produced by crossing tetraploid male and diploid female have similar abnormal embryo rates to controls (Myers and Hershberger, 1991), suggesting some of the abnormalities observed in other studies might be due to the treatment rather than to triploidy itself. For example, McKay et al. (1992) found that fry mortalities were higher for triploids produced by heat shock of all three genetic hybrids produced (*Salvelinus fontinalis* × *Salmo trutta*). In rainbow trout, thermal shock induced higher mortality and deformity rates than pressure treatment (Haffray et al., 2007). The rate of fry abnormalities was not different between pressure treatment and diploid control (2.8 % vs. 1.9 %), but was significantly higher in fry from thermal treatment (11.7 %).

More recently, Fjelldal and Hansen (2010) studied the prevalence of deformities in four full-sib groups of diploid and triploid Atlantic salmon smolts subjected to a pressure shock. Deformity prevalence was higher in triploid (1-3 %) vs. diploid (0-1 %) with the trunk region of the vertebral column being the predominant region of deformity in triploids (vertebra 24 most often affected). Therefore, by provision of optimal hatchery incubation temperature (< 8 °C) and triploidy induction protocols, comparable survival and deformity prevalence to diploids might be achievable in triploid salmonids.

In diploid fish dietary deficiency in phosphorus affects bone mineralisation in rainbow trout (Fontagne et al., 2009) and phospholipid concentration has been shown to affect the spinal malformation rate in sea bass (Cahu et al., 2003).

Phosphatidylinositol in particular has shown to prevent skeletal deformities in marine fish (Cahu et al., 2009). Therefore, by providing optimised nutrition observed deformities in many of the studies may be reduced. To date, triploids have been fed on diets formulated for diploid fish which presumes that triploids have the same dietary requirements as diploids. However there is growing evidence to suggest that triploids may have different/increased dietary requirements than diploids. Triploid Atlantic salmon exhibited differences in body composition, with higher energy and lipid levels and lower moisture and ash levels when fed various amount of dietary phosphorous, however there were no effects of ploidy on feed digestibility and bone mineral content (Burke et al., 2010). However, it has been suggested triploid salmon may need higher dietary phosphorous to support faster growth and prevent skeletal malformation (Fjelldal & Hansen, 2010). Diploid and triploid Atlantic salmon siblings were fed with a phosphorous dietary level that was deficient, within or higher than the estimated requirements of diploids from first feeding to smolt stage. It was found that higher occurrence of vertebral deformity for the low and medium diet, and a low and equal deformity incidence in diploids and triploids for the high phosphorous diet (Fjelldal et al., 2012a). In addition, vertebral bone mineral content at smolting was equal for the low and high diets however was lower in triploid than diploids for the diet with a phosphorous level estimated within the requirement range in diploids. This suggests among the nutritional factors, optimal phosphorus nutrition during specific periods, for example after transfer to sea water, appears to be critical for development of deformity at later stages (Fjelldal et al., 2012b). In freshwater, ash and phosphorous levels in the scales and carcasses of triploid rainbow trout were significantly lower after two weeks in fish fed a phosphorus deficient diet compared to those fed a phosphorus sufficient diet (Le Luyer et al., 2014). In diploid fish

nutritional factors such as deficiencies of micronutrients (e.g. zinc and vitamin B2) and amino acids (e.g. methionine, cysteine, and tryptophan) have been shown to increase cataract risks in salmonids (Tacon, 1993; Waagbo et al., 2003). Studies in diploid Atlantic salmon indicated that observed cataracts were significantly lower in smolts fed a histidine-enriched diet (Breck et al., 2005). In addition, cataract severity was directly related to the dietary histidine level during warm temperature and fast growth and that feeding with histidine-supplemented diets mitigated against later cataract outbreaks in adult diploid Atlantic salmon (Waagbo et al., 2010). Similarly in triploid Atlantic salmon which can grow faster than diploids, histidine supplementation is required to mitigate further cataracts occurrence in triploids during seawater grow-out (Taylor et al., 2014b).

Currently there is limited knowledge regarding the genetic and molecular mechanisms underlying the triploidy status. The close study of gene regulation will provide an insight into how physiological and endocrine mechanisms are influenced by ploidy, thus improving the overall understanding of how these mechanisms regulate growth and deformity prevalence. Recently, differences in gene expression were observed between diploid and triploid rainbow trout during fasting and re-feeding periods and indicate that regulation of physiological mechanisms are affected by ploidy and may contribute to observed differences in nutrient partitioning and growth rate (Cleveland & Weber, 2013). Gene expression in the triploid *Squalius alburnoides* found that compensation mechanisms exist, reducing transcript levels to the diploid state, suggesting gene silencing in one of the three alleles. It was found that not all of the haplome is inactivated and the allelic expression patterns differ between genes and between different tissues for one of the same gene (Pala et al., 2008). Interactions were observed among transgene dosage, maternal effects,

developmental stage and ploidy on growth and endocrine parameters in GH transgenic Coho salmon (Devlin et al., 2014). These findings illustrate the importance to better understanding the process and implications of genome regulation, silencing and dosage effects in triploids.

Currently, the majority of the studies indicate increased prevalence of skeletal deformity in triploids which is a welfare concern. The mechanisms instigating this phenomenon have not been full elucidated. However, recently, evidence suggested that triploids have different dietary requirements to diploids and that nutritional deficiency is a major cause of skeletal malformations. Sub-optimal husbandry and induction protocols influence deformity prevalence and reduce survival in triploids. However, it is possible that through improved selection, triploid specific diets, optimising husbandry/induction protocols while also furthering the understanding of genome regulation in triploids may provide mitigation against deformity prevalence. While there is very limited knowledge on deformity prevalence in triploid brown trout, the optimisation of current triploidy induction protocols and husbandry conditions must be achieved to ensure triploid brown trout are not predisposed to increased deformity as seen in other species.

2.8 Behaviour and post stock out performance of triploids

The behaviour of triploid fish appears to be different than diploids, with a more subdued behaviour in general (Table 2). It is possible that the reduction in cell numbers and morphology and the reduced levels of steroid hormones in triploids may impact on the behaviour of triploids (Benfey, 1999). In diploid fish, increased levels of androgens (testosterone and 11-ketotestosterone) have been linked to territorial dominance and aggression in male African cichlid fish *Haplochromis*

burtoni (Francis et al., 1992). To further validate these results, dominant males were gonadectomised, which resulted in pronounced reductions in steroids and aggression. In contrast to this, female triploid fish have been shown to have lower levels of sex steroids than diploids. For example, plasma levels of gonadotropin (GtH-II), testosterone (T) and Estradiol-17β (E_2) in female triploid Indian catfish (*Heteropneustes fossilis*) were significantly lower during the annual reproductive cycle than diploids (Tiwary et al., 2001). This clearly confirmed the sterile status of triploid females. However, as triploid males are still able to develop testes, normal steroid levels have been found in most fish species except in the Indian catfish (Tiwary et al., 2004). It is possible that reduced circulating hormones makes triploids less aggressive than diploids. For example, triploid Atlantic salmon appears to be less aggressive than diploids, and exhibit more severe fin damage than diploids when communally reared (Carter et al., 1994). Feeding behaviour was investigated by O'Keefe & Benfey (1999) between diploid and triploid brook trout in tanks. There was no difference between ploidy in growth, food consumption and conversion rates in either separate or mixed ploidy groups. Triploid fighting fish, *Betta splendens*, also showed a reduced aggressive behaviour (erection of fins or opercula, air gulping, undulating movements, striking and biting) than diploids, which was suggested to be due to their sterility status (Kavumpurath & Pandian, 1992).

Czesney et al. (2002) showed that triploid juvenile saugeyes (♂ walleye *Stizostedion vitreum* x ♀ sauger *Stizostedion canadense*) exhibited less efficient foraging than diploids, which could reduce growth, increase risk of predation, and decrease their survival after stocking. Wagner et al. (2006) found there was no significant difference between diploid and triploid rainbow trout in aggressive behaviour (chases and counter attacks) using size-matching pairs (diploid-triploid).

Table 2. Summary of studies reporting the behaviour of sterile salmonids in the wild.

Species	Triploidy treatment	Main Effects	Authors
Coho salmon			
Oncorhynchus kisutch	Hormone treatment	Lack of homing behaviour. Males display sexual behaviour in wild	Solar et al. (1986)
Rainbow trout			
Oncorhynchus mykiss	Heat	Display of site fidelity	Bridger et al. (2001)
	Heat	Same capture rate by anglers	Dillion et al. (2000)
	Heat	Same post-stocking survival	Wagner et al. (2006)
Rainbow trout			
Oncorhynchus mykiss	Heat	Triploid survival similar to diploids	High and Meyer (2009)
	Heat	AF3N higher return rates in alpine lakes	Koenig et al. (2011)
Atlantic Salmon			
Salmo salar	Heat/pressure	Males triploids migrate, females do not	Wilkins et al. (2001)
	Heat/pressure	Inability to interbreed	Cotter et al. (2000)

Also, the aggression of triploid Chinook salmon was significantly less than diploids or mixed groups of diploids/triploids in tank trials (Garner et al., 2008). Similarly, hormonally-sterilised diploid and triploid coho salmon released into the wild showed reduced homing instinct when in seawater, although males exhibited sexual characteristics which could interfere with wild conspecifics (Solar et al., 1986). Other studies have examined the release of Atlantic salmon and rainbow trout into the wild. Wilkins et al. (2001) studied seawater migrations of Atlantic salmon in Ireland and indicated that male triploids returned to natal rivers in similar proportions to diploids, whereas the majority of triploid females did not. Similar results were found in the return rate of triploid Atlantic salmon to the coast, and freshwater which was substantially reduced, and Cotter et al. (2000) indicated that low reproductive capabilities reduced risks of inbreeding with wild fish. Recent results suggested triploid Atlantic salmon have altered brain morphology, having a significantly smaller olfactory bulb but a larger cerebellum and telencephalon than diploids (Fraser et al., 2012a). As the olfactory bulb has a number of important functions in

teleosts, such as in feeding and anti-predator behaviour (Wisenden, 2000) and migration (Mitamura et al., 2005), it is possible that reduced olfactory function could result in decreased survival through impaired behavioural responses. The ploidy effects on the cerebellum and telencephalon correspond to previous reports of altered aggression and foraging ability in triploids and should be examined in terms of their effects on ability of triploids to cope in the wild (Fraser et al., 2012a).

There are studies, which indicate the potential use of triploids as a genetic conservation method within freshwater fisheries. Triploid grass carp (*Ctenopharyngodon idella*) has been used to control hydrilla (*Hydrilla verticillata*) infestations in southern U.S. reservoirs for several decades (Kirk et al., 2014). In salmonids, mixed sex triploid rainbow trout were used to observe the relative recapture rates of triploids post stocking in Idaho, U.S. (Dillion et al., 2000). Overall returns were not significantly different between ploidies and additionally the time to harvest did not differ between groups. Koenig et al. (2011) suggested fisheries managers should consider stocking all-female rainbow trout (AF3N) in alpine lakes. This approach is a low-risk option of maintaining stocks within alpine lakes while minimising the impacts on native stocks. AF3N returned in higher proportions than mixed sex 3N and diploid 2N rainbow trout. High & Meyer (2009) used radio transmitters to track triploid “catchable size” rainbow trout in an Idaho stream and demonstrated an average life expectancy of 14.3 days, and 30 days post-stocking 85 % of trout were presumed dead. Wagner et al. (2006) compared the growth performance of triploid rainbow trout in three Idaho rivers at 138 days post-stocking and found no significant difference in mean weight between or feed conversion ratio of either ploidy.

It appears from available studies that triploid fish exhibit less aggressive behaviour than diploids when observed in feeding and behavioural studies. This may be related to the circulating hormone levels, which are reduced in triploid females in comparison to diploids females. Post stock-out studies indicate AF3N can survive equally or better than diploids in alpine lakes and should be considered by fishery managers as a low risk stocking option. Currently, studies on behaviour and post stock out performance are limited to rainbow trout and Atlantic salmon, and further work is needed in brown trout to determine if similar observations exist. Only once such knowledge is available can the potential for triploid brown trout in freshwater fisheries within the United Kingdom be effectively utilised.

3. Aims of the thesis

The main aims of such a study are:

1. To test a number of protocols (test pressure intensity and duration), and the effect of egg quality on triploid yields in brown trout (optimisation).
2. Develop a protocol for triploid verification of brown trout using erythrocyte indices, flow cytometry and microsatellite markers (assessment)
3. To assess the impact of triploidy on growth performance, deformity prevalence and welfare in brown trout.
4. To observe behavioural interactions (feeding preferences and aggression) between diploid and triploid brown trout within an experimental flume.
5. Test the physiological response of diploid and triploid brown trout to sub-optimal environmental conditions (temperature).
6. To transfer working protocols (standardised) and guidance to the UK trout and freshwater fishery sectors.

CHAPTER 2

RESEARCH ARTICLE

OPTIMISATION OF TRIPLOIDY INDUCTION IN BROWN TROUT (*Salmo trutta L.)*

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Keywords: brown trout, *Salmo trutta* L, triploid, pressure shock, flow cytometry, yield

Abstract

Inland fisheries contribute substantially to the economies of England and Wales. Many trout fisheries rely partly or entirely on stocking to maintain catches. Given that farmed trout often differ genetically from their wild counterparts, wild trout could be at risk of reduced fitness due to interbreeding or competition with farmed fish. As a preventative measure, the UK Environment Agency has implemented the "National Trout and Grayling Strategy" which will only give consent to the stocking of rivers and some still waters with sterile, all-female triploid brown trout. In order to produce an optimised hydrostatic pressure induction protocol three experiments were conducted to (1) determine the optimal timing of application post-fertilisation, (2) define optimal pressure intensity and duration of the shock and (3) study the effect of temperature (6–12 °C) on triploid yield. Triploid rate was assessed using two different ploidy verification methods (blood smear and flow cytometry). Results indicated high survival to yolk sac absorption stage (69.2–93.6%) and high triploid yields (82.5–100%) from the range of treatments applied. Furthermore, no significant differences in triploid rates were shown when shock timings and durations were adjusted according to the temperature used. In all treatments deformity prevalence remained low (<1.8%) during incubation up to yolk sac absorption (~550 degree days post hatch). Overall, this study showed that the optimised pressure shock for the induction of triploidy in brown trout delivering high survival and 100% triploid rate (a prerequisite to brown trout restocking) is a shock with a magnitude of 10,000 psi (689 Bar) applied at 300 centigrade temperature minutes (CTM) for at least 5 min duration. The study also validated blood smear and flow cytometry as simple and accurate ploidy assessment techniques for brown trout.

1. Introduction

Angling pressure and lack of local recruitment due to anthropogenic factors have reduced wild brown trout (*Salmo trutta* L.) populations in many areas, leading to stocking with farmed fish (Aprahamian et al., 2003). Concerns have been raised about the damage stocking farm-reared trout can have on wild populations of brown trout (Jug et al., 2005; Hansen et al., 2009). Farmed trout differ genetically from their wild counterparts and in this respect, wild trout are at risk due to interbreeding and introgression with farmed fish, which may lead to loss of local adaptation and reduced fitness; other potential effects include competition for resources and transfer of disease (DeWald and Wilzbach, 1992).

Restocking of brown trout has taken place for over a century, with generally negative impacts on genetic integrity, although these appeared to be generally smaller than expected due to reduced fitness and limited spawning success of stocked fish (Hansen et al., 1995; Heggenes et al., 2006; Apostolidis et al., 2008; Wollenbaek et al., 2010). Introgression is also positively correlated with the numbers of fish stocked and the duration of the stocking (Arias et al., 1995; Garcia-Marin et al., 1999; Araguas et al., 2004; Wollenbaek et al., 2010). Therefore, as a conservation effort, the Environment Agency (EA) has introduced new legislation to protect wild brown trout in England and Wales, where 4 million anglers spend around £3 billion per annum (EA, 2004). Under the umbrella of the National Trout and Grayling Fisheries Strategy, brown trout stocked into all but totally enclosed waters with no significant natural brown trout populations will be with either sterile all-female brown trout or brown trout from breeding programmes using locally sourced broodfish by 2015 (EA, 2009). All-female triploids could prevent genetic introgression through sterility,

thus contributing to good ecological management of freshwater bodies. However, protocols to induce triploidy must be reliable and result in high survival and low deformity prevalence (for economic reasons) as well as 100 % triploid rate.

Triploids have been commercially or experimentally produced in many fish and shellfish species by pressure shock (Johnstone et al., 1991; Gillet et al., 2001; Garner et al., 2008; Chiasson et al., 2009), heat shock (Benfey, 1984; Rougeot et al., 2003), cold shock (Piferrer et al., 2003; Dias de Silva et al., 2007) or chemical shocks using cytochalasin B in oysters (Grendreau and Grizel 1990; Hand and Nell, 1999). Currently there are reliable triploid induction protocols for some commercially important salmonid species (e.g. *Salmo salar, Oncorhynchus mykiss*). However, there is limited published information on triploid induction in brown trout. Only two publications indicated that triploid brown trout can be produced by heat shocks (Arai and Wilkins, 1987; Crozier and Moffett, 1989); reduced survivals were obtained suggesting that an optimised heat shock had not been identified, or that heat shock gives less consistent success than hydrostatic pressure shock (HP), which is recognised as a more reliable technique to produce triploid fish (Piferrer et al., 2009). Brydges and Benfey (1991) published results of a preliminary study on the induction of triploidy in brown trout using HP shocks and demonstrated high triploid rates could be achieved using HP shock, however, optimised conditions were not identified during the range of experiments.

Triploids are expected to be sterile due to interference with gametogenesis, however the levels of induced sterility vary between the sexes. Most studies on the reproductive physiology of triploid fishes indicated females are sterile, while males can produce some post meiotic cells which are generally aneuploid and in low

numbers (Benfey, 1999). For this reason, all-female triploid fish have been employed for restocking purposes of brown trout, as in other salmonid species (Wilkins et al. 2001). However, there is concern that the current HP protocol developed by the British Trout Association (BTA) and the Environment Agency (EA) has not been fully optimised (HP of 11,000 psi, 300 CTM for 7 mins: British Trout Association and UK Environment Agency, 2006). Therefore there is a clear need for further optimisation to validate a reliable protocol to assist the brown trout aquaculture and fisheries sector within the UK.

HP shocking is dependent on three main variables, which influence the effectiveness of the shock: timing, intensity and duration of the shock (Felip et al., 1997; Piferrer et al., 2009). HP shocks consist of a period of increased hydrostatic pressure applied to recently fertilised eggs to suppress the second polar body extrusion; however the exact mechanisms are not well documented. There is a "time window" during which triploidy can be induced and optimised: the application of a shock outside this window results in reduced triploid rate and/or survival (Hussain et al., 1991). Timing of the HP shock is intrinsically linked to the rate of embryonic development, which varies with temperature; therefore the optimum timing of the shock is dependent on the egg incubation temperature before the shock is applied.

The aims of this study were to (1) determine the optimal timing of application of the HP shock, (2) define the optimal intensity and duration of the HP shock and (3) determine the effect of incubation temperature on triploid induction. Results in terms of survival, triploid rate, triploid yield and deformity prevalence were compared against the current triploid induction protocol used in UK trout farms.

2. Materials and Methods

2.1 Experimental Design

The study includes three experiments performed consecutively during the brown trout spawning season (October-December) from 2009 to 2011. The experiments incorporate key variables of triploid induction in brown trout including the timing, intensity and duration of the pressure shock. All experiments were conducted under flow-through hatchery conditions at the Institute of Aquaculture (University of Stirling) freshwater facility (Howietoun hatchery). Water supplied from the Coulter burn was at a temperature of 6.2 ± 2.3 °C (mean ± SD). All gametes for 2009 and 2010 were obtained from mature 2-3 year-old brown trout broodstock of Howietoun strain (mean weight 2,096.8 ± 127.3 g), while in 2011 broodstock from Loch Leven strain (mean weight 866.5 ± 59.1 g) maintained at Howietoun fishery were used.

During the spawning season, brown trout ovulation was monitored twice weekly by manual inspection of the broodstock. In each experiment, fish were anaesthetised using tricaine methane sulphonate (MS-222, 80 ppm) and eggs/milt was manually stripped. Eggs were collected from individual females (mean egg batch weight: 660.5 ± 111.0 g) then sub-divided into equal batches and kept cool until treatment. Prior to artificial fertilisation, eggs and milt were protected from contact with water to prevent water hardening and sperm activation. Eggs were deemed to be of good quality on the basis of their colour, size and consistency (Mansour et al., 2007). Spermatozoa motility was checked under a light microscope prior to fertilisation by activating a sample from each male with water. The batches of eggs (~500/batch) were fertilised in plastic beakers with pooled milt (0.3 ml / batch) and mixed gently for 30 seconds. 200 ml of hatchery water (10 °C for experiments 1 and

2, 6 -12 °C for experiment 3) was added to the eggs, which were then placed in a water bath at the same temperature for 1 minute. The water was then discarded to remove both ovarian fluid and milt. 400 ml of hatchery water at the same temperature was then added to the fertilised eggs, which were placed back into the water bath until required for pressure shocking. All batches of eggs used as diploid controls were handled in the same way as experimental triploid batches, except that no HP shock was applied. Timing of the shock post-fertilisation is temperature-dependent and therefore expressed as Centigrade Temperature Minutes (CTM). Therefore, for the application of a shock at 300 CTM at 10 °C, the following timings were used: at 275 CTM the pressure vessel was filled with 1.5 L of 10 °C water. At 285 CTM, eggs were taken out of the water bath and directed into the 1.5 L custom made hydrostatic pressure vessel supplied by the University of Stirling's Institute of Aquaculture. Then at 295 CTM the pressure was gradually increased. For each pressure shock, a 30 second period of increasing pressure was allowed to reach the shock pressure (10, 000 psi - pounds per square inch; 68.95 MPa), which was maintained for the shock duration (4-7 minutes) then a 30 second period was allowed to decrease pressure to ambient after the shock. Once the atmospheric pressure was reached, eggs were taken out of the vessel and put into a bucket of freshwater at ambient temperature for an hour to both acclimate them to the hatchery water temperature and to facilitate the water hardening process. Multiple HP shocks were conducted by fertilising individual egg batches every 15 minutes (British Trout Association and UK Environment Agency, 2006). Standard hatchery troughs, with lightproof hoods, were used to incubate the fertilised eggs. Sub-samples were removed from each egg batch and counted to volumetrically estimate egg numbers prior to laying down (25 ml

corresponding to ~125 eggs). Monitoring of survival, weight, deformity prevalence and triploid rates took place at ~550 degree days post hatch (referred to elsewhere in the text as yolk sac absorption stage, YSA) prior to the addition of exogenous feed. Skeletal deformities were determined by scoring of "visible" deformities from each treatment including scoliosis, lordosis, kyphosis, spiralled tails and Siamese (double headed) fish according to Bruno and Poppe (1996). Deformities were removed daily from hatch to YSA and euthanized in MS-222, and then fixed in 10% neutral buffered formalin pots (100 ml). Thereafter, deformity prevalence was determined by adding the total number of deformities and dividing by the total of hatched embryos within the treatment, giving deformity prevalence (%) from hatch to YSA.

2.2 Experiment 1: Optimal timing of pressure shock

The eggs from four individual females were separately aliquoted into ten equal batches and held in plastic beakers (~500 eggs/batch). Two replicate batches of eggs from each female were not subjected to an HP shock and served as controls (pooled in Table 1). The first control was set up at the start and the second at the end of the series of HP shocks. Controls were placed at the start and end of the series in the incubation trough, with replicates reversed in order of treatments. The remaining eight batches of eggs from each female were subjected to an HP shock applied by placing one batch of eggs at a time into the pressure vessel. One batch of eggs from each lot was subjected to the induction protocol recommended by the Environment Agency (EA) (11,000 psi HP shock for 7 minutes at 300 CTM, batch Trip 8). The other seven batches of eggs from each lot were subjected to a 10,000 psi HP shock for 5 minutes with start times ranging from 275 CTM to 425 CTM at 10 ° C (Trip 1 -

7, 25 CTM increments). The experiment therefore included a total of 40 egg batches (four females x 10 groups per female) (Table 1).

2.3 Experiment 2: Pressure intensity and duration

Ten different combinations of pressure shocks were tested on four different egg batches. Brown trout eggs (Howietoun strain) were collected and pooled into 4 batches (2 females and 4 males per batch). Batches of eggs (~500 eggs/treatment) from each lot were subjected to one of nine combinations of three pressure intensities (9,500, 10,000 or 10,500 psi) and three shock durations (4, 5 or 6 mins) at 300 CTM, or the EA shock protocol, all at 10 °C (total of 10 HP treatments / batch). Two replicate batches of eggs from each batch (n = 8) were not subjected to a HP shock and used as controls. The experiment therefore included a total of 48 egg batches (4 batches x 12 groups) (Table 1).

2.4 Experiment 3: Optimal induction temperature

Four different water temperatures and HP shocks (with shock parameters adjusted for temperature) were tested on three egg batches stripped from Loch Leven strain brown trout. Eggs were collected and pooled into 3 batches (2 females per batch). Batches of eggs were subdivided (~250 eggs/treatment) and subjected to HP of 10,000 psi applied using water temperatures of 6, 8, 10 and 12 °C (T6, T8, T10 and T12). The HP shocks all started at 300 CTM (at 50, 37.5, 30 and 25 MPF for T6, T8, T10 and T12 respectively) for durations of 8 min 20 s, 6 min 15s, 5 min and 4 min 10 s respectively. The experiment included 60 egg batches: three replicate HP (triploid)

and two controls (diploid) were created for each temperature from each egg batch (Table 1).

2.5 Ploidy determination

Experiment 1: At yolk sac absorption (YSA) twenty fry were randomly sampled from each incubator, killed by overdose of phenoxyethanol (Sigma) and blood smears prepared by severing the caudal peduncle. A drop of blood was spread on a glass slide and allowed to air dry before being fixed by a 10 min immersion in 100 % methanol. The fixed blood smears were then stained in 6 % Giemsa (Sigma) for 10 min (no cover-slip was added). The major axis of 15 - 20 erythrocyte nuclei were determined from each blood smear using a light microscope fitted with a 1 mm graticule marked at 1 µm intervals.

Experiments 2 and 3: 50 alevins were randomly sampled from each incubator for ploidy verification by flow-cytometry analysis. Whole alevins were prepared as previously described by Lecommandeur et al. (1994). Each alevin was over-anaesthetized (with phenoxyethanol), the yolk sac removed with a scalpel blade and discarded. The alevin was then blast frozen in liquid nitrogen (-196 ^{o}C), five alevins per 1.5 ml tube. The samples were then kept at -70 ^{o}C until required for analysis. After thawing, individual alevins were placed into 0.5 ml of phosphate buffered saline (PBS) and homogenised using a mechanical pestle. Samples were then centrifuged at 2000 rpm (447 g) for 5 mins and the supernatant removed before 0.5 ml of 0.1 % Triton-X100 and 0.1 % sodium citrate buffer was added to the pellet (Lecommander et al., 1994). Samples were vortexed then passed through a 15 µm filter before being stained with 10 µl of propidium iodide (10 mg/ml). After 30

minutes incubation at 4 ^{o}C, relative DNA contents were measured using a Becton Dickinson Facscalibur flow cytometer. Histogram measurements were calculated using peak fluorescence on an FLA-2 photomuliplier tube. Instrument linearity was checked using internal reference beads (Nile Red 2.5 µm, BD Biosciences, San Jose USA). Diploid and triploid individuals were easily identified by flow cytometry measurements of the cell suspension. Histogram peaks with fluorescence >27.5 % above that of control diploids were deemed triploid (Fig. 1).

2.6 Statistical analysis

Statistical analysis was performed using Minitab v15 statistical software (Minitab, Coventry, UK). All data sets were checked for normality using the Kolmogorov-Smirnov test and homogeneity of variance using Bartlett's test, and arcsine-transformed when normality and/or homogeneity of variance was not confirmed. Differences in mean survival rates, mean weights, mean Erythrocyte Nuclear Length (ENL), mean triploid yields and deformity rate were tested using a GLM ANOVA with post-hoc analyses carried out using Tukey's Multiple Comparison tests. Differences in mean triploid rates were calculated with InStat 3 (GraphPad Software, Inc) using a Kruskal-Wallis non-parametric ANOVA. For ploidy analysis by flow cytometry, all data were collected and analysed using Cellquest Version 3.3 (BD Biosciences, San Jose USA). Differences in mean peak fluorescence were determined using a Kruskal-Wallis non-parametric test. Triploid yields were calculated as the percentage survival of triploids relative to controls multiplied by the triploid rate of each treatment. All statistical comparisons were performed using a significance level of 5 % ($p < 0.05$). Results are presented as mean ± SEM.

3. Results

3.1 Experiment 1: Optimal timing of pressure shock

Eggs from all HP treatments showed high survival to YSA (ranging from 69.2 ± 7.4 to 74.8 ± 8.8 %) with no significant differences among treatments (Table 1). Survival in the EA treatment (69.2 ± 7.4 %) was lower than all other treatments, although differences were not significant. ENL were significantly smaller in the diploid controls than in all HP treatments (diploid 5.50 ± 0.03 μm and triploid 7.70 ± 0.09 μm, $P < 0.05$). Mean triploid rates (Table 1) and yields were high and not significantly different in all HP treatments (Fig. 2a), with the highest values for mean triploid rate and yield in the Trip 2 treatment (300 CTM: 100.0 ± 0.0 % and 93.7 ± 0.0 %, respectively) and the lowest in the Trip 7 treatment (425 CTM: 82.5 ± 1.3 % and 81.2 ± 1.6 %, respectively).

Deformity rate was low in all HP treatments, ranging from 0.1 ± 0.3 to 1.8 ± 1.2 % and was not significantly different between controls and any of the HP treatments, although the Trip 2 (300 CTM, 0.1 ± 0.3 %) and EA (1.8 ± 1.2 %) treatments were significantly different (Table 1). No significant differences were observed among treatments in fry weight at YSA.

3.2 Experiment 2: Pressure intensity and duration

All treatments displayed high survival rates up to YSA (ranging from 87.3 ± 4.1 to 93.0 ± 1.8 %) with no significant differences among treatments and controls (Table 1). Mean triploid rates were high in all HP treatments, ranging from 93.6 ± 5.5 % to 100.0 ± 5.5 % (Table 1). Treatments Trip 5, 8, 9 and 10 displayed 100 % triploid rates, however these were not statistically different from other treatments. Coefficient

of variation (CV) of fluorescence peaks from muscle homogenate for diploid and triploid was 4-10 % when stained with PI (same for experiment 3). Triploid yields ranged from 98.7 ± 2.2 % (Trip 5) to 89.4 ± 7.9 % (Trip 1) (Fig. 2b), but did not differ significantly among treatments.

Deformity rates ranged from 0.5 ± 0.3 % (Dip 1) to 1.7 ± 0.7 % (EA), but there were no statistically significant differences between treatments. There was no significant difference between treatments in fry weight at YSA.

3.3 Experiment 3: Induction temperature

In HP groups, survival to YSA ranged from 72.1 ± 1.7 % (Trip 6) to 80.1 ± 2.3 (Trip 10) but without significant differences (Table 1). No significant differences were observed between diploid and HP treatments. Mean triploid rates were high in all HP treatments, ranging from 96.6 ± 3.3 % (Trip 12) to 100 % (Trip 10), with no significant differences. However, triploid yield was significantly higher in the Trip 10 treatment (120.3 ± 5.2 %) than in the other treated groups, largely due to lower survival in the relevant diploid control group (Fig. 2c). Deformity rates were low in all HP treatments and not significantly different between diploids and HP treatments. No significant differences between treatments in fry weight at YSA were observed.

Table 1. Mean survival (%) and deformity rate (%) at yolk sac absorption (YSA) assessed in experiment 1, 2 and 3. Superscripts denote significant differences between treatments ($P < 0.05$). All data are expressed as mean of the replicates ± SEM (n = ~250-500/replicate). The timing of the shock is indicated by CTM (Centigrade Temperature Minutes) and MPF (Minutes Post Fertilisation).

Experiment	Treatments	Pressure (PSI)	Timing (CTM)	Timing (MPF)	Duration (mins)	Mean Triploid Rate (%)	T °C	Mean Survival at YSA (%)	Deformity Rate (%)
1	Dip 1	Diploid			0	0.0 ± 0.0	10.0	78.3 ± 5.1^{a}	0.5 ± 0.8^{ab}
	Trip 1	10,000	275	27.5	5	98.8 ±1.3^{a}	10.0	74.8 ± 8.8^{a}	0.8 ± 0.6^{ab}
	Trip 2	10,000	300	30	5	100 ± 0.0^{a}	10.0	72.0 ± 11.4^{a}	0.2 ± 0.3^{a}
	Trip 3	10,000	325	32.5	5	94.9 ± 3.5^{a}	10.0	74.4 ± 12.1^{a}	0.4 ± 1.0^{ab}
	Trip 4	10,000	350	35	5	94.7 ± 5.3^{a}	10.0	71.6 ± 15.3^{a}	0.7 ± 1.3^{ab}
	Trip 5	10,000	375	37.5	5	96.3 ± 1.4^{a}	10.0	73.3 ± 9.8^{a}	0.4 ± 0.3^{ab}
	Trip 6	10,000	400	40	5	96.3 ± 3.8^{a}	10.0	73.7 ± 9.6^{a}	0.4 ± 0.6^{ab}
	Trip 7	10,000	425	42.5	5	82.5 ± 1.3^{a}	10.0	72.6 ± 6.8^{a}	1.0 ± 1.9^{ab}
	(Trip EA)	11000 (EA)	300	30	7	95.4 ± 1.7^{a}	10.0	69.2 ± 7.4^{a}	1.8 ± 1.2^{b}
2	Dip 2	Diploid			0	0.0 ± 0.0	10.0	93.7 ± 2.8^{a}	0.5 ± 0.3^{a}
	Trip 1	9,500	300	30	4	97.3 ± 2.3^{a}	10.0	87.3 ± 4.1^{a}	0.5 ± 0.2^{a}
	Trip 2	10,000	300	30	4	97.5 ± 2.2^{a}	10.0	92.4 ± 0.6^{a}	0.6 ± 0.1^{a}
	Trip 3	10,500	300	30	4	93.6 ± 5.5^{a}	10.0	91.2 ± 2.1^{a}	0.6 ± 0.2^{a}
	Trip 4	9,500	300	30	5	95.7 ± 3.7^{a}	10.0	91.8 ± 2.1^{a}	0.6 ± 0.3^{a}
	Trip 5	10,000	300	30	5	100.0 ± 0.0^{a}	10.0	92.1 ± 1.5^{a}	0.8 ± 0.4^{a}
	Trip 6	10,500	300	30	5	99.3 ± 0.6^{a}	10.0	88.7 ± 3.2^{a}	0.9 ± 0.3^{a}
	Trip 7	9,500	300	30	6	98.0 ± 1.0^{a}	10.0	91.1 ± 3.0^{a}	0.8 ± 0.3^{a}
	Trip 8	10,000	300	30	6	100.0 ± 0.0^{a}	10.0	89.8 ± 4.7^{a}	0.4 ± 0.1^{a}
	Trip 9	10,500	300	30	6	100.0 ± 0.0^{a}	10.0	93.0 ± 1.8^{a}	0.8 ± 0.2^{a}
	(Trip EA)	11,000	300	30	7	100.0 ± 0.0^{a}	10.0	88.6 ± 3.3^{a}	1.7 ± 0.7^{a}
3	Dip 6	Diploid			0	0.0 ± 0.0	6.0	75.9 ± 1.8^{a}	1.2 ± 0.3^{a}
	Dip 8	Diploid			0	0.0 ± 0.0	8.0	79.9 ± 0.5^{a}	0.9 ± 0.3^{a}
	Dip 10	Diploid			0	0.0 ± 0.0	10.0	67.6 ± 1.5^{a}	1.3 ± 0.2^{a}
	Dip 12	Diploid			0	0.0 ± 0.0	12.0	78.2 ± 0.4^{a}	1.3 ± 0.5^{a}
	Trip 6	10,000	300	50	8 min 20 s	97.8 ± 2.2^{a}	6.0	72.1 ± 1.7^{a}	2.1 ± 0.5^{a}
	Trip 8	10,000	300	37.5	6 min 15 s	96.7 ± 1.9^{a}	8.0	79.1 ± 2.7^{a}	1.0 ± 0.4^{a}
	Trip 10	10,000	300	30	5 min	100.0 ± 0.0^{a}	10.0	80.1 ± 2.3^{a}	1.0 ± 0.3^{a}
	Trip 12	10,000	300	25	4 min 10 s	96.7 ± 3.3^{a}	12.0	78.8 ± 3.6^{a}	1.3 ± 0.5^{a}

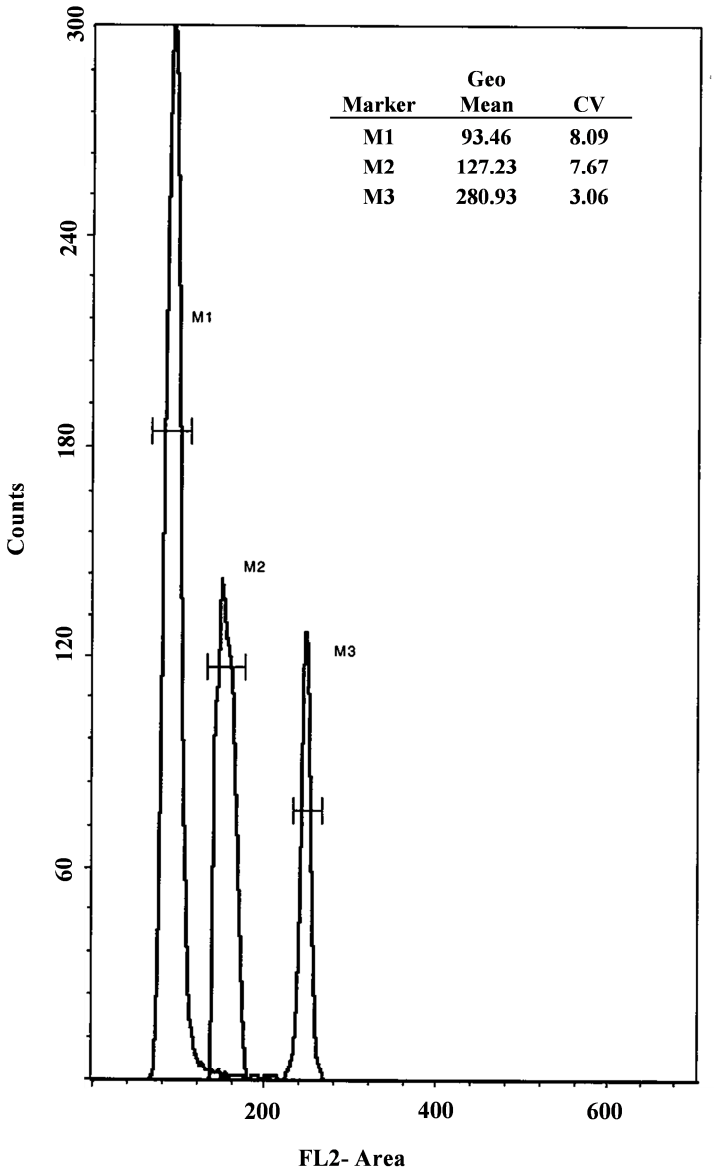

Figure 1. Flow cytometric DNA distribution of *Salmo trutta* diploid (M1) and triploid (M2) alevin and reference beads (M3) following staining with 10 mg/ml propidium iodide (PI). DNA values are reported in arbitrary units expressed as fluorescence channel numbers (FL2-Area). A total of 10,000 cells analysed per sample with mean peak fluorescence 91.92 ± 0.75 and 126.34 ± 0.82 respectively for diploid and triploid. DNA histogram statistics are indicated for all three markers.

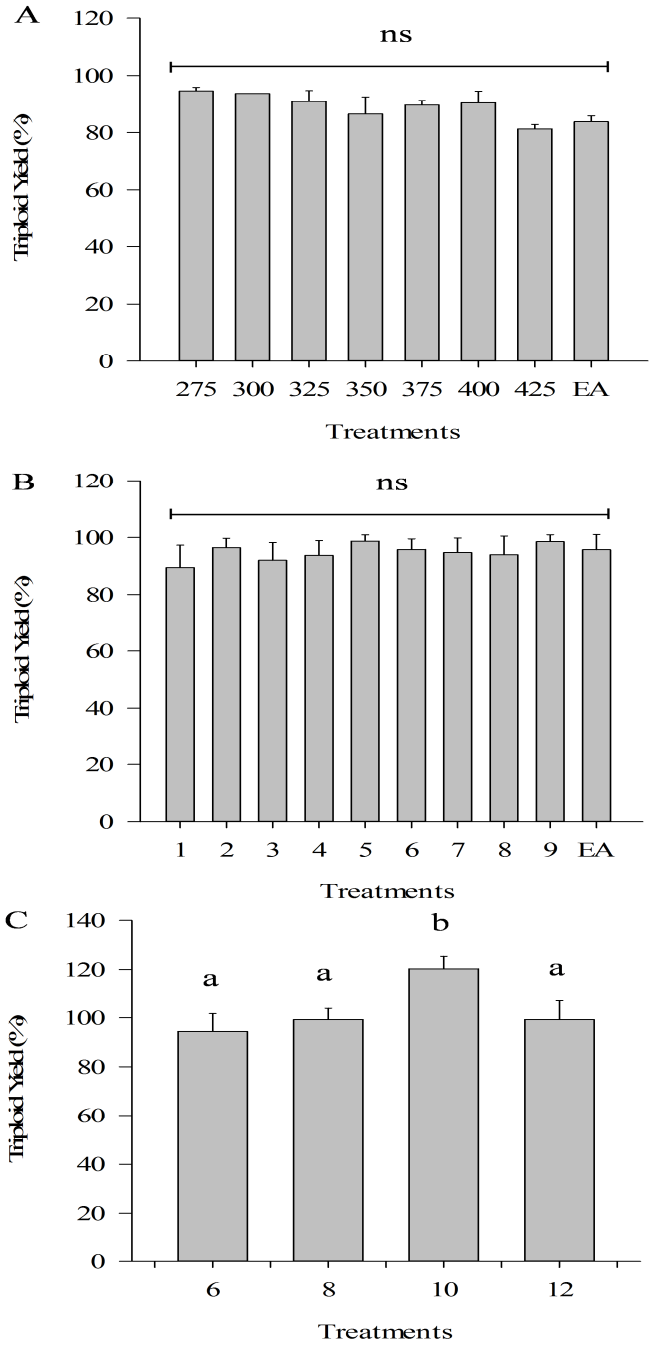

Figure 2. Mean (± SEM) triploid yield (%) within brown trout treatment groups. A: Experiment 1: 10,000 psi HP shock for 5 minutes with start times ranging from 275 to 425 CTM and EA shock (11,000 psi HP shock for 7 minutes at 300 CTM). B: Experiment 2, where a combination of pressure intensities (9,500, 10,000 or 10,500 psi) and shock durations (4, 5 or 6 mins) were applied at 300 CTM (see Table 1 legend and text for full description of each shock) in comparison to the EA shock protocol. C: Experiment 3, where a 10,000 psi shock was applied at 300 CTM at water temperatures of 6, 8, 10 or 12 °C (durations of 8 min 20 s, 6 min 15s, 5 min and 4 min 10 s, respectively). Different letters indicate significant differences between groups ($P < 0.05$); ns indicate the lack of significant differences.

4. Discussion

In the present study, the three key variables involved in HP shocks were tested to optimise the process of triploid induction in brown trout. The results indicate a wide window in the timing at which high triploid rates (> 90 %) can be obtained in brown trout (275 – 400 CTM). Also, brown trout subjected to a combination of pressure intensities (9,500, 10,000 or 10,500 psi) and duration of the shock (4, 5, 6 or 7 mins) applied at 300 CTM exhibited high survivals irrespective of the magnitude and duration of the HP. Similar results with regards to the plasticity of eggs of other salmonids to the timing of HP shock have been reported in the literature. Gillet et al. (2001) obtained triploid yields of 82 to 100 % in Arctic charr (*Salvelinus alpinus*) by applying HP shock of 10,050 psi for 5 mins duration applied at 240, 320 and 400 CTM, however, the same study showed that application of the shock marginally outside this window greatly affected triploid yields. Chiasson et al. (2009) indicated that an HP shock of 9,500 psi for 5 mins at 210 CTM, which appears marginally outside the window described by Gillet et al. (2001), was effective at inducing 100 % triploid rate: mean triploid yield was 83 %, in eight full-sib families of Arctic charr. In Coho salmon (*Oncorhynchus kisutch*), HP shocks of 9,000-12,000 psi for 4 mins were effective at inducing triploidy. It was shown that triploid rates of 100 % could be achieved by application of HP shock at 210 CTM, however survival was inversely correlated with the intensity of the treatment, and therefore triploid yields were low at higher pressure intensities (Teskeredzic et al., 1993).

The intensity and the duration of the HP shock have also been shown to affect the success of triploidy induction in salmonids. Chourrout (1984) reported that induction of triploidy in rainbow trout through HP shocks using 6,000 psi for 8

minutes applied at 376 CTM post fertilisation (9.4 °C) resulted in low survivals. Survival and triploid rates were however higher for a HP shock of 7,000 psi for 3 - 5 minutes duration applied at 376 CTM. In Atlantic salmon, Benfey and Sutterlin (1984) reported that high triploid yields could be achieved by applying a 10, 150 psi HP shock for 3 - 6 minutes at 200 CTM. Deviations in the HP shock duration or intensity resulted in 100 % mortality prior to hatching. This said, in the present study, high survivals at YSA in triploids indicated no adverse effect of pressure intensities ranging from 9,500 to 11,000 psi and duration from 4 to 7 minutes. Also, although broodstock used in the present study were of similar origin the potential for differential responsiveness to the triploid induction process (intensity, duration and temperature) cannot be overlooked. In addition, the potential long-term effects were not addressed in the present study and should be further investigated. Similarly high survival and triploid rates were obtained in Chinook salmon (*Oncorhynchus tshawytscha*) using a HP shock of 10,000 psi, duration of 5 mins, applied at 300 CTM (Johnson et al., 2004).

Early development in warm water fish is generally much quicker, which greatly impacts on the timing of the shock. For example, Linhart et al. (2001) reported triploid induction in European catfish (*Siluris glanis*) using a HP shock of 8,534 psi for 4 minutes (70 CTM). However triploid yields were reduced when the same shock was applied at 105 CTM as opposed to 52.5 CTM. High triploid yields can be achieved in Nile tilapia (*Oreochromis niloticus*) by using a HP shock of 7,500 – 8,500 psi for 2 minutes applied at 224 – 280 CTM (Hussain et al., 1991). According to this study, when the treatment was applied prior to 224 CTM, the triploid yield was zero, and triploid yield was also significantly reduced when HP shocks were applied between

308 – 336 CTM. The importance of appropriate pressure level to inhibit polar body extrusion has been described in grass carp, *Ctenopharyngodon idella* and the highest triploid yields were obtained when HP shocks of 7,000 to 8,000 psi were applied at 4 MPF for 1 or 2 mins (88 – 99.2 CTM) (Cassani and Caton, 1986). Lower survival rates were obtained when the pressure was increased to 9,000 psi. Triploid rates quickly dropped with the decrease of pressure below 6,000 psi suggesting the inability of low-pressure shocks to inhibit the retention of the second polar body during meiosis II (Cassani and Caton, 1986).

Observations in non-salmonid temperate species suggest the time window in which triploidy can be induced appears much narrower. This is probably due to a range of factors including the faster embryonic developmental times: e.g. 245 HPF (hours post fertilisation) from fertilization to hatch in cod *Gadus morhua* (Hall et al., 2004) *vs.* 1,200 HPF (505 – 535 degree days) in Atlantic salmon (Taylor et al., 2011). It has also been suggested that decreased survival in triploids in some non-salmonid temperate species (e.g. turbot, *Scophthalmus maximus*) is not only due to the triploid status itself but also the effects of mechanical handling of fragile fertilised eggs during the induction process (Piferrer et al., 2000).

The wide window during which HP shock can induce triploidy in brown trout and the plasticity in the pressure magnitude as well as duration of the shock is a great advantage to farmers in complying with the Environment Agency's all-female triploid stocking policy. In addition, moderate variations in timing and pressure application would still result in high triploid rates. This said, although mean triploid rates were high and not significantly different between treatments in all experiments, 100 % triploid rate was only achieved in the 300 CTM treatment for experiment 1, treatments

5, 8 and 9 in experiment 2 and T10 °C for experiment 3 meaning a shock of at least 10,000 psi, applied at 300 CTM post fertilisation for a minimum of 5 mins duration is required at 10 °C. While in the present study mixed sex triploids were produced, due to sex-reversed broodstock (neomales) not being available, it would not be expected that any impact on triploid induction process would occur using sperm from either XY males or XX neomales.

It appears from the data presented in the present study that the best shock in terms of highest survival, highest triploid rates and lowest deformity prevalence is a HP shock of 10,000 psi for 5 mins at 300 CTM. In contrast the shock endorsed by the EA (11,000 psi for 7 mins duration at 300 CTM) appeared to slightly increase deformity prevalence (significantly so in experiment 1 compared to shock of 10,000 psi for 5 mins at 300 CTM), probably as a result of the increased pressure and duration of the treatment. The high survival and triploidy rate obtained in most HP shocks tested highlight the plasticity of brown trout eggs with regards to pressure magnitude, duration and timing post-fertilization, which contrast with other fish species (Cassani and Caton, 1986; Linhart et al., 2001). This clearly justifies the use of HP shocks in brown trout, as in other salmonid species, over temperature shocks which can be seen as a simpler technique but are generally less reliable, probably due to the difficulty of applying a controlled change of temperature homogenously on an egg batch (Piferrer et al., 2009).

Once the shock timing, duration and intensity have been optimised in a given species at a set temperature, then the remaining variable that may affect the HP treatment efficiency is ambient temperature variation. The pre-shock incubation temperature is responsible for the developmental rate of the fertilised eggs,

determining when the HP shock application should be applied to affect meiosis II. Maintaining the 10 – 11 ^{o}C pre-shock incubation temperature recommended by the EA could be difficult in farming conditions especially when producing out-of-season eggs as air temperature, in-flow water temperature and the efficiency/reliability of water heating systems may all have an effect on the temperature of the pre-shock water. The results from the present study suggest that triploidy can be induced effectively at a range of temperatures by adjusting the timing and duration of the HP shock. Although the majority of previous studies were performed at a temperature of 10 °C, the present study confirmed the relationship between temperature and embryonic development in brown trout within the 6 to 12 °C range. Embryonic development times at a range of temperatures (5 - 20 °C) have been shown not to differ significantly between species (zooplankton, salmonids and amphibians) when defined in terms of degree days (Gillooly and Dobson, 2000). This indicates that temperature and embryonic development are intrinsically linked in many ectothermic organisms. The present study shows a HP shock of 10,000 psi for 5 mins at 300 CTM is effective at producing high triploid yields at a range of temperatures that would allow for some variation in the pre-shock incubation temperature recommended by the EA (10 °C), either above (12 °C) or below (6 °C; 8 °C). This would clearly facilitate the use of HP shocks in farming conditions.

In order to test for the efficiency of the HP shocks, ploidy verification has to be performed. A variety of techniques including blood smears and erythrocyte nuclei length assessment, karyotyping, nucleolar-organising region (NOR) analysis, flow cytometry DNA-analysis, electronic estimation of ploidy using a Coulter counter and genotyping of microsatellite DNA markers have all been successfully used in earlier

studies in a range of species, including Atlantic salmon (Benfey and Sutterlin, 1984; Friars et al., 2001; Taylor et al., 2011), grass carp (Wattendorf, 1986), chinook salmon (Johnson et al., 2004), Caspian salmon, *Salmo trutta caspius* (Kalbassi et al., 2009) and Arctic charr (Gillet et al., 2001), and also sea bass *Dicentrarchus labrax* (Felip et al., 1997; Peruzzi and Chatain, 2000) and European catfish (Linhart et al., 2001) among others. Blood smears and erythrocyte nuclei length assessment is often preferred for ploidy verification for simplicity and economic reasons (Piferrer et al., 2009). However, flow cytometry analysis is very sensitive, a large number of cells can be analysed (10,000 per analysis), samples can be kept frozen for extended periods and many samples can be analysed over a day. Importantly, this technique can give an early assessment of the ploidy status of a population as analyses can be done on very young embryonic stages with a very small amount of tissue needed (Thorgaard et al., 1982; Lecommandeur et al., 1994). Flow cytometry analysis based on measurements of the DNA content of cells via fluorescence of stained DNA has already been used with success for ploidy analysis in rainbow trout, Atlantic salmon, Arctic charr, Chinook salmon and Caspian salmon (Thorgaard et al., 1982; Solar et al., 1984; Friars et al., 2001; Gillet et al., 2001; Johnson et al., 2004, Kalbassi et al., 2009) although this technique has not yet been implemented commercially within the brown trout industry. As confirmed by the present study, flow cytometry to determine ploidy status could become a very useful screening technique to ensure farmers comply with the new legislation in the UK. Validation at a commercial scale is now required.

The present study showed that high triploid yields can be achieved in brown trout by using a 10,000 psi HP shock of 5 minutes duration applied between 275 – 425 CTM post fertilisation at 10 ^{o}C. The high survival/triploid rates obtained using a range

of HP (9,500 - 11,000 psi) and duration (4, 5, 6 or 7 mins) highlights the plasticity of brown trout eggs, which offer flexibility to triploid brown trout producers. Further research is now required to assess triploid brown trout during the grow out phase to determine if there are any long term effects of suboptimal HP shocks beyond the YSA stage. Also, and of particular importance, are investigations into the competition for resources post stock-out including the feeding and behaviour responses of triploids, which will assist in assessing the ecological impact of stocking triploids into natural environments.

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CHAPTER 3.

RESEARCH ARTICLE

ASSESSING THE EFFECTIVENESS OF TRIPLOIDY INDUCTION IN BROWN TROUT (*SALMO TRUTTA*) USING IMAGE ANALYSIS, FLOW CYTOMETRY AND MICROSATELLITE MARKERS.

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Keywords: brown trout, DNA, diploid, triploid, ploidy, verification, nuclear length, flow cytometry, microsatellite.

Abstract

The production of sterile brown trout (triploid) for stocking freshwater sport fisheries is a management goal to reduce the risk of introgression associated with stocking reproductively competent trout within England and Wales. In 2015, the UK Environment Agency will only give consent to stocking all-female triploid brown trout (*Salmo trutta*) or brown trout from locally-sourced broodstock. Therefore, methods for assessing triploidy in brown trout are required by the aquaculture and governmental sectors to ensure compliance with legislation. Traditional methods used to verify triploidy in brown trout are labour-intensive and not convenient for screening large numbers of commercial trout. Therefore, the present study assessed the applicability of microsatellite markers to resolve ploidy levels in brown trout along with the two traditional methods routinely used e.g. red blood cell measurements and flow cytometry. Brown trout were selected from a commercially available sibling group of all-female diploid and triploid (hydrostatic pressure shocked) trout stock. DNA profiling of 22 genetic markers selected from the literature indicated that 18 microsatellites successfully discriminated at least one locus exhibiting trisomy (three alleles present) in triploids, while diploids displayed a maximum of two alleles. Red blood nuclear and cell measurements and flow cytometry confirmed the findings of the genetic technique however may be less applicable commercially due to the cost, being labour-intensive and display some size overlap in nuclear and cell minor measurements. In addition, by refining the current panel of markers, six of the most highly polymorphic centromere distant microsatellites identified would be sufficient to correctly identify ploidy levels with 100 % accuracy within the population tested. The microsatellites identified may provide aquaculture and freshwater fisheries with a

more accurate, economic and non-invasive tool for assessing ploidy in brown trout than traditional techniques, ensuring compliance with freshwater stocking legislation.

1. Introduction

Freshwater fisheries are commercially important to the economies of England and Wales and are recognized for their high quality sport fishing. With an estimated 430,000 trout fishing anglers, many fisheries stock farmed brown trout (*Salmo trutta L.*) to ensure the sustainability of these fisheries (Spurgeon et al., 2001). Most trout are released into fisheries when reaching 0.5 Kg weight with an average of 750,000 trout stocked per annum (EA, 2007). However it has been recognized that the large scale stocking of farmed trout could pose threats to wild populations through competition, predation and introgression (De Wald and Wilzbach, 1992). While fertile brown trout have been used in stocking for many generations it is thought the impacts on genetic integrity of wild fish are generally smaller than expected due to reduced fitness and limited spawning success of farm-reared stocked fish (Hansen et al., 1995; Heggens et al., 2005; Apostolidis et al., 2008, Wollebaek et al., 2010). Indeed, the genetic impact of the farmed population has been reported to be lower than expected suggesting that survival of farmed trout is lower than in wild brown trout (Hansen, 2002). On the other hand, genetic introgression has been shown to positively correlate with the numbers of fish stocked and the duration of the stocking (Arias et al., 1995; Garcia-Marin et al. 1999; Araguas et al., 2004; Wollenbaek et al., 2010). Therefore, freshwater fisheries management have introduced measures to alleviate the risk of genetic introgression from farmed to wild trout.

The Environment Agency (EA) has introduced legislation to protect remaining wild brown trout populations in England and Wales (EA, 2009). Brown trout stocked into all but totally enclosed freshwaters with no significant natural brown trout populations will be with either sterile all-female triploid brown trout or brown trout raised from breeding programmes using locally-sourced brood fish within that catchment (EA, 2009). This decision has followed an extensive consultation process evaluating its contribution towards the good

ecological management of freshwater bodies forecast for 2015, as defined in the EU Directive 2000/60/CE of 23/10/2000. Three steps were taken prior to implementing the legislation, which included: a bibliography survey, a risk analysis of stocking with triploids and common-garden experiments. The implementation plan was conducted over a 5 year period with the overall objective to reduce the use of farmed diploid brown trout by 30 % in 2010 and by 50 % in 2013 until their replacement with all-female triploids in 2015 (Piferrer et al., 2009). The use of genetically sterile all-female triploids may prevent genetic introgression through sterility thus contributing to the good ecological management of freshwaters. However, this freshwater management plan hinges on having robust measures to assess and verify ploidy status of farm-reared brown trout.

Triploids have been commercially or experimentally produced in many fish and shellfish species by hydrostatic pressure, heat, cold or chemical shocks (reviewed by Piferrer et al., 2009). In brown trout, triploids have been produced by heat (Arai and Wilkins, 1987; Crozier and Moffat, 1989) and pressure shock (Brydges and Benfey, 1991; Preston et al., 2013). Triploids possess three sets of chromosomes due to second polar body retention and therefore have larger nuclei to facilitate the increase of genomic content (Benfey, 1999). However, the production of triploids either commercially or experimentally requires an accurate method to validate ploidy status and the success of the induction process.

Studies have shown that triploids can be identified by chromosome preparations (Pradeep et al. 2011); red blood cell (RBC) measurements (Dorafshan et al. 2008; Taylor et al. 2011), coulter counter (Johnson et al. 1984), and flow cytometry (Peruzzi et al. 2005; Preston et al., 2013), and genotyping of microsatellite markers (Krieger et al., 1999), however the reliability and accuracy of some techniques have come into question (Johnson et al. 1984). RBC nuclear and cellular size is a simple and cost-effective method however this technique is

time consuming and not possible on small fish due to blood collection requirements. DNA content determination by flow cytometry analysis is very sensitive and allows large numbers of cells to be analysed (between 5,000 and 15,000 per analysis) per sample. Using flow cytometry, samples can be kept frozen for extended periods, small amounts of sample material are needed for the analysis and several hundred samples can be analysed every day on early eyed-eggs (Thorgaard et al., 1982; Leucommandeur et al., 1994). However, some of the techniques have disadvantages due to costly equipment, destructive nature of the sampling process and the speed of the procedure not being suitable for screening large numbers of experimental or commercial groups. Therefore, an economic and robust method for the determination of ploidy status in brown trout at any post embryonic stage of development would be advantageous to the aquaculture and freshwater fisheries to ensure compliance with legislation.

The development of genomic resources has facilitated the identification of highly polymorphic and centromere distant microsatellite markers for resolving ploidy levels in aquaculture species (Hernández-Urcera et al., 2012; Nie et al., 2014). Parameters such as polymorphism and accuracy of genotyping are essential to assess the potential robustness of microsatellites for ploidy verification (Hernández-Urcera et al., 2012). To date, a large number of microsatellites have been characterised in brown trout (Keenan et al., 2013). In addition, the development of a genetic map in the species and the localisation of the centromeres allow positioning of markers to be established and an estimation of recombination frequency to centromeres (Gharbi et al., 2006; Hernández-Urcera et al., 2012). If marker-centromere distances are high, crossing over will occur during prophase I of meiosis and therefore if both parents do not share any alleles for specific markers, triploid individuals will display trisomy and be identifiable as having three alleles at a given locus (Hernández-Urcera et al., 2012). Highly

polymorphic telomeric microsatellite markers may therefore offer a molecular tool to validate triploidy in brown trout at any post-embryonic stage of development and ensure compliance with freshwater fisheries legislation. The aims of this study were to assess an accurate, cost-effective, and user-friendly molecular tool for resolving ploidy levels in farmed brown trout. The performance of this genetic technique was compared with traditional techniques including RBC measurements and flow cytometry.

2. Materials and Methods

2.1 Fish stock sample collection

Commercial batches of all-female diploid (2N) and triploid (3N) brown trout eggs were supplied from a commercial brown trout farm located in Devon England, United Kingdom. On 23rd November 2011, brown trout brood fish were stripped and eggs and milt (~4 mL) (XX males: neomales) collected and pooled into one large batch for fertilisation. The batch was divided into two equal groups, with one subjected to hydrostatic pressure shock to induce polar body retention (3N), while the other received no shock (2N), giving two groups (all- female diploid (2N) and triploid (3N) c. 2500 per group). Triploidy was induced in one batch using a hydrostatic pressure shock of 689 Bar at 300 centigrade temperature minutes (CTM) for 5 minutes duration using a customized pressure chamber (Preston et al., 2013). Fertilised ova were then water-hardened and each ploidy group was disinfected and stocked in isolation into a separate hatchery trough for incubation under controlled temperature using spring water at each hatchery (at 10 ± 0.5 °C). Then on the 22nd December 2011, eyed eggs (300 degree days post fertilisation) were shipped from Devon, England to the University of Stirling's freshwater facility (Niall Bromage Freshwater Research facility, Buckieburn, Scotland UK) where they were maintained within a closed recirculation unit at 10.0 ± 0.5 °C.

Fish were hatchery-reared in 2 m diameter (1500 L) production tanks (1 tank/ ploidy group) until the time of sampling. The experimental work was reviewed by the University of Stirling Ethics Committee and conducted under U.K. Home Office licence.

2.2 Sample collection

On the 27 April 2012, 96 trout fry (n = 48 / tank) were sampled from each of the stock tanks (mean ± SD weight diploid: 1.35 ± 0.3 g, length: 46.54 ± 3.6 mm; triploid 1.10 ± 0.3 g, length: 46.54 ± 4.1 mm). Fish were euthanased in MS-222 (150 mg/L) then the caudal peduncle was removed by surgical ablation to expose the caudal vein. A small drop of blood (~50 µL) was placed onto a glass slide cover then a blood smear created using the techniques previously described by Pradeep et al. (2011). The remaining fry were blast frozen in liquid nitrogen (-196 ^{o}C), one trout fry per 1.5 ml microfuge tube. The samples were then kept at -70.0 ± 5.0 ^{o}C until required for analysis. Prior to DNA content analysis by flow cytometry a small section of caudal fin was removed and stored in 70 % ethanol until later DNA extraction.

2.3 RBC measurements

After air-drying, slides were fixed in 100% methanol for 10 minutes then placed into Giemsa stain (10 %) for 10 minutes. Excess Giemsa was washed off with distilled water before being place in a fume hood and allowed to dry overnight. Erythrocyte minor and major axes and their nuclei were measured at 400x magnification using image capture (ImagePro Software 4.1). A total of 20 randomly chosen blood cells per slide were measured to the nearest 0.01 µm. Surface area (S) was calculated as $S = ab\pi/4$ where *a* and *b* are the major and the minor axis of the cell or the cell nucleus, respectively (Dorafshan et al. 2008). As the erythrocytes

of brown trout are ellipsoid, the cell and nucleus volumes were calculated using the formula V = (4/3) AB^2 where A and B are the major and minor axes of the cell and nucleus (Uzunova, 2002).

2.4 DNA content by flow cytometry.

A 250 mg sample of tissue was dissected from each individual frozen fry (caudal section) and placed into 0.5 ml of phosphate buffered saline (PBS) and homogenised using a mechanical pestle. Samples were then centrifuged at 2000 rpm (447 g) for 5 minutes and the supernatant removed before 0.5 ml of 0.1 % Triton-X100 and 0.1 % sodium citrate buffer was added to the pellet (Lecommandeur et al. 1994). Samples were vortexed then passed through a 15 µm filter before being stained with 10 µl of propidium iodide (Sigma Aldrich, 10 mg/ml). After 30 minutes incubation at 4 ^{o}C in the dark, relative DNA contents were measured using a Becton Dickinson Facscalibur flow cytometer. Acquisition panels were set up in Cellquest version 3.3 using the default parameters of Forward Scatter (FSC) on the x-axis and Side Scatter (SSC) on the y-axis for dot, and FL2-Area on the x-axis and counts on the y axis for histogram acquisition plots respectively. The parameter was selected according to the fluorochrome that was used, with FL-2 detecting the orange fluorescence emitted by Propidium iodide (Sigma Aldrich, Poole England catalogue no: 556463). A total of approximately 10,000 events were collected per sample and using the polygonal region tool cell populations of interest was gated. Statistical outputs were generated using Cellquest version 3.3 software, with relative fluorescence measurements determined between ploidy for geometric mean and peak fluorescence respectively. Controls were animals that were not subjected to hydrostatic pressure shock (diploid). Additionally, whole blood samples from known diploid brown trout were used as a quality control. Instrument range and sensitivity were analysed using calibrite

beads ™ before use (BD Biosciences, San Jose USA). Instrumental and staining variability was checked by analysis of a single sample held on ice in a dark cool box at the start and end of each sample processing period (~2 hrs.).

2.5 Microsatellite genotyping.

DNA was obtained from caudal fin tissue by a standard salt extraction method (Aljanabi & Martinez, 1999). Following spectrophotometric quantification (Nanodrop), samples were standardised to 5 ng / µL in 5 mM Tris pH8.5. Two optimised marker panels; comprising 13 & 9 loci respectively (Table 1) were screened for each sample on a 96 capillary ABI-3730XL DNA analyser (Applied Biosystems, Paisley, UK). The cycling conditions for the two multiplex PCRs were as follows: (95◦ C for 15 min) ×1 cycle (95◦ C for 45 s, 55◦ C for 1min 30 s and 72◦ C for 1min) ×5 cycles, (95◦ C for 45 s, 57◦ C for 1min 30 s and 72◦ C for 1min) ×22 cycles, (60◦ C for 30 min) ×1 cycle. Each multiplex reaction consisted of 1 µl of template DNA (*c.* 5 ng), 0.15 μM of each primer, 1.75 µl of PCR mastermix (Qiagen Multiplex PCR Kit) and double-distilled H2O as required to make a final volume of 3.5 µl. PCRs were carried out in 96 well microtitre plates and were overlain with 10 µl of mineral oil to prevent evaporation. Following PCR, amplified fragments were subsequently diluted 1:10 with double-distilled H_2O and 1 µl of this dilution was added to 9 µl of HiDi formamide (Life Technologies) mixed with Gene Scan 600-LIZ (Life Technologies), as per standard ABI 3730xl genotyping protocol. Following PCR amplification allelic expressions were determined using GeneMapper 4.1 (Applied Biosystems). For the purpose of this study it was decided to infer trisomy at a locus only when three different sized alleles were observed. While it is sometimes possible to infer trisomy from double peaked chromatograms, by

comparison of peak intensities, this is a less reliable approach requiring extremely well characterised loci (Hernández-Urcera et al., 2012).

2.6 Statistical analysis

Red blood cell measurements were examined for normality and homogeneity of variance using Anderson Darling and Kolmogorov-Smirnov tests respectively. Mean RBC values between ploidy were compared using a one-way ANOVA or a Mann-Whitney test when normality and/or homogeneity of variance were not confirmed. For flow cytometry, differences in peak and geometric mean fluorescence intensity are presented in arbitrary units expressed as channel numbers. Differences between ploidy were analysed using Cellquest version 3.3 (BD Biosciences, Oxford England) and a non-parametric Mann-Whitney test. All statistical tests were performed using Minitab v16 with a significance level of $P < 0.05$. Results in all figures are presented as mean ± SD.

3. Results

3.1 RBC measurements

Among the control group, all fish sampled were identified as diploid (n = 48) from the combination of cellular and nuclear sizes measured. The hydrostatic pressure-shocked group exhibited 100 % triploid rate. The mean values of the cellular major and minor axes were 1.30 and 1.10 times larger in triploid than in diploid trout ($P < 0.001$). Cellular surface area was 1.47 larger in triploid than diploids ($P < 0.001$). The mean values of the nuclear major and minor axes were 13.6 and 1.19 times larger in triploids than in diploids ($P < 0.001$). Nuclear surface area of triploids was 1.63 times larger than in diploids ($P < 0.001$). Nucleus and cell volume was 1.49 and 2.05 times bigger in triploids ($P < 0.001$) (Table 2)

Table 1. Details of the twenty two polymorphic microsatellite markers in the present study.

Panel/locus	Primer sequence (5'–3')	No. of alleles	Allele size range	Reference
Ssa85	F: NED-AGGTGGGTCCTCCAAGCTAC R: gtttACCCGCTCCTCACTTAATC	9	100-125	O'Reilly *et al* . (1996)
One102-a	F: NED-GGGATTATTCTTACTTTGGCTGTT R: gtttCCTGGTTGGGAATCACTGC	3	160-176	Olsen *et al* . (2000)
One102-b	F: NED-GGGATTATTCTTACTTTGGCTGTT R: gtttCCTGGTTGGGAATCACTGC	18	174-280	Olsen *et al* . (2000)
Ssa406UOS	F: NED-ACCAACCTGCACATGTCTTCTATG R: gtttGCTGCCGCCTGTTGTCTCTTT	38	418-580	Cairney *et al* . (2000)
CA054565a	F: VIC-TCTGTGGTTCCCGATCTTTC R: gtttCAACATTTGCCTAGCCCAGA	1	101-120	Vasemagi *et al* . (2005b)
ppStr2	F: PET-CTGGGGTCCACAGCCTATAA R: gtttGAGCTACAACCTGATCCACCA	40	173-350	Kennan *et al.* (2013)
Ssa416	F: FAM-TGACCAACAACAAACGCACAT R: gtttCCCACCCATTAACACAACTAT	4	100-160	Cairney *et al* . (2000)
One103	F: AATGTTGAGAGCTATTTCAATCC R: GATTGATGAATGGGTGGG	89	167-447	Olsen *et al.* (2000)
SsaD48	F: GAGCCTGTTCAGAGAAATGAG R: CAGAGGTGTTGAGTCAGAGAAG	48	203-457	King *et al.* (2005)
Cocl-Lav-4	F: VIC-TGGTGTAATGGCTTTTCCTG R: gtttGGGAGCAACATTGGACTCTC	8	145-170	Rogers *et al.* (2004)
One9uASC	F: CTCTCTTTGGCTCGGGGAATGTT R: GCATGTTCTGACAGCCTACAGCT	7	150-200	Scribner *et al.* (1996)
CA048828	F: VIC-GAGGGCTTCCCATACAACAA R: gtttGTTTAAGCGGTGAGTTGACGAGAG	35	245-326	Vasemagi *et al.* (2005b)
CA053293	F: PET-TCTCATGGTGAGCAACAAACA R: gtttACTCTGGGGCATTCATTCAG	9	140-171	Vasemagi *et al.* (2005a, b)
BG935488	F: gttTGACCCCACCAAGTTTTTCT R: NED-AAACACAGTAAGCCCATCTATTG	10	110-165	Vasemagi *et al.* (2005b)
SsaD71	F: NED-AACGTGAAACATAAATCGATGG R: gtTTAAGAATGGGTTGCCTATGAG	19	170-257	King *et al.* (2005)
SaSaTAP2A	F: gtttGTCCTGATGTTGGCTCCCAGG R: NED-GCGGGACACCGTCAGGGCAGT	13	280-447	Grimholt *et al.* (2002)
MHCI	F: PET-AGGAAGGTGCTGAAGAGGAAC R: gtttCAATTACCACAAGCCCGCTC	14	110-150	Grimholt *et al.* (2002)
Ssa410UOS	F: gtttGGAAAATAATCAATGCTGCTGGTT R: PET-CTACAATCTGGACTATCTTCTTCA	31	168-326	Cairney *et al.* (2000)
ppStr3	F: FAM-CTGACCGCTGCACACTAA R: gtttGGCTCTAATCGACTGGCAGA	5	115-175	Kennan *et al.* (2013)
CA060177	F: VIC-CGCTTCCTGGACAAAAATTA R: gtttGAGCACACCCATTCTCA	12	234-315	Vasemagi *et al.* (2005b)
Ssa197	F: VIC-GGGTTGAGTAGGGAGGCTTG R: gttTGGCAGGGATTTGACATAAC	11	120-176	O'Reilly *et al.* (1996)
One108	F: VIC-GTCATACTACTCATTCCACATT R: gtttACACAGTCACCTCAGTCTATTC	38	371-518	Olsen *et al.* (2000)

Table 2. Red blood cell nuclear and cellular data for diploid and triploid brown trout respectively. Data presented as mean ± SD.

Dimensions	**Ploidy**		**Ratio [diploid:triploid]**	**Significance level**
	Diploid (n=48)	**Triploid (n=48)**		
Nuclear major axis (µm)	6.11 ± 0.72	8.34 ± 1.0	1.36	$P < 0.05$
Nuclear minor axis (µm)	4.46 ± 0.67	5.30 ± 0.80	1.19	$P < 0.05$
Nuclear surface area (μm^2)	21.42 ± 4.32	34.73 ± 7.04	1.63	$P < 0.05$
Cellular major axis (µm)	20.50 ± 2.32	26.66 ± 2.84	1.30	$P < 0.05$
Cellular minor axis (µm)	11.74 ± 1.32	12.95 ± 1.41	1.10	$P < 0.05$
Cellular surface area (μm^2)	184.54 ± 41.97	271.36 ± 42.07	1.47	$P < 0.05$

3.2 *DNA content by flow cytometry.*

The coefficients of variation (CV) of fluorescence peaks from the nuclei of diploid and triploid brown trout were regularly 2-5 % (diploid: 3.98 ± 0.24; triploid: 4.54 ± 0.37 %) when stained with the fluorochrome propidium iodide (PI). The geometric mean was 1.51 times greater in triploids than in diploids (diploid: 94.53 ± 2.41; triploid: 143.10 ± 3.40, $P < 0.001$). Mean peak channel number was 1.53 times greater in triploid than in diploid trout (diploid: 93.65 ± 2.49; triploid: 143.56 ± 3.42) (Figure 1).

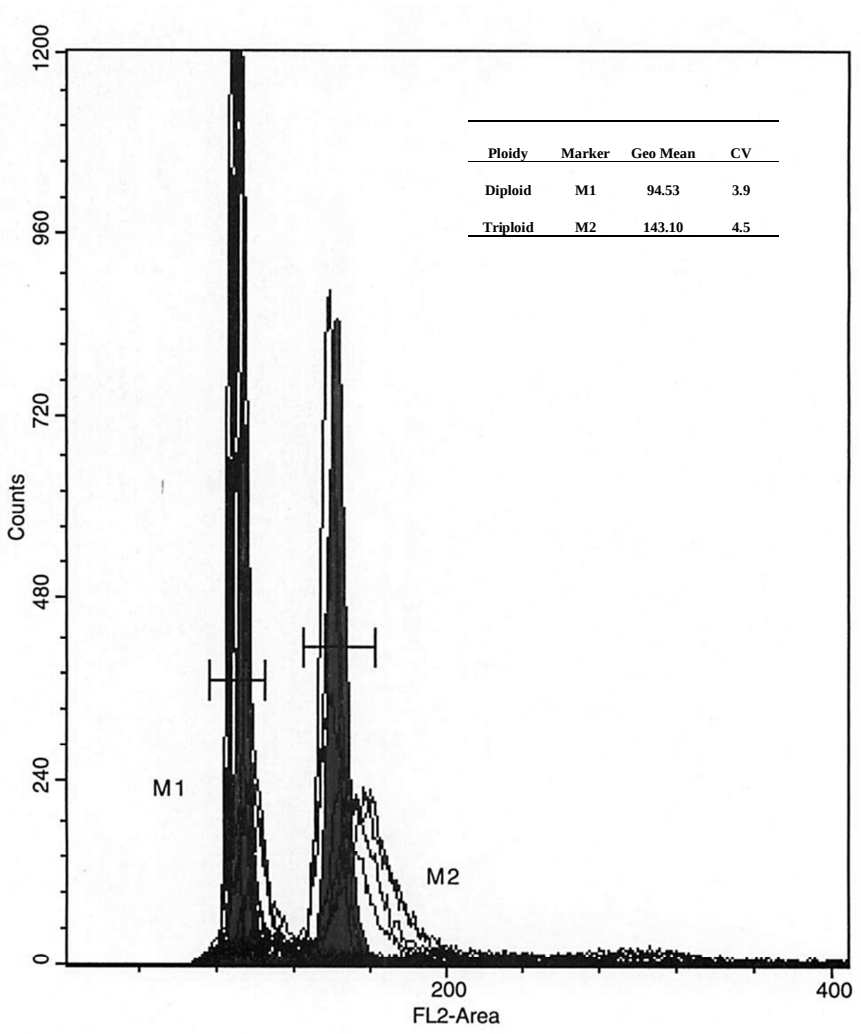


Figure 1. Overlay histogram plot of nuclear DNA content of diploid (n =10) and pressure shocked triploid (n=10) brown trout measured by flow cytometry (diploid: M1, triploid: M2). DNA values (geometric mean) are reported in arbitrary units expressed as fluorescent channel numbers (FL2-Area) along with relative coefficient of variation.

3.3 *Marker selection and loci scoring*

From the original 22 candidate loci assessed, 18 met the specified selection criteria with at least one locus exhibiting trisomy (three alleles present). All pressure-shocked trout were observed with three different sized alleles at a given loci (n = 48, Table 3A & B). Chromatograph interpretation indicated that triploids displayed 4.44 ± 1.89 (mean ± S.D.) loci displaying trisomy using the chosen markers (Figure 2).

A subset of three particularly informative markers were apparent in this study, SSa410UOS, One 102-b and CA048828 which positively identified (3 different sized alleles) 79.17 %, 54.17 % and 39.58 % of triploid individuals, respectively. The chromatograms confirming trisomy were clearly identifiable as traces with three discernible peaks (each corresponding to a specific allele). Diploids trout exhibited a maximum of two alleles for any one locus for all the microsatellite markers studied. In addition, by refining the current microsatellite markers to include six of the most informative loci, (Ssa410UOS; One 102-b; CA048828; Ssa406UOS, Ssa410UOS and One 103), would be sufficient to detect triploids with 100 % accuracy in the population studied.

The microsatellite locus One108 revealed no information and was excluded from any further analysis. Panel information of microsatellite loci including the sample number (BT 1-96), the number of alleles observed at each locus, and ploidy of individuals screening during this study are shown in Appendix II.

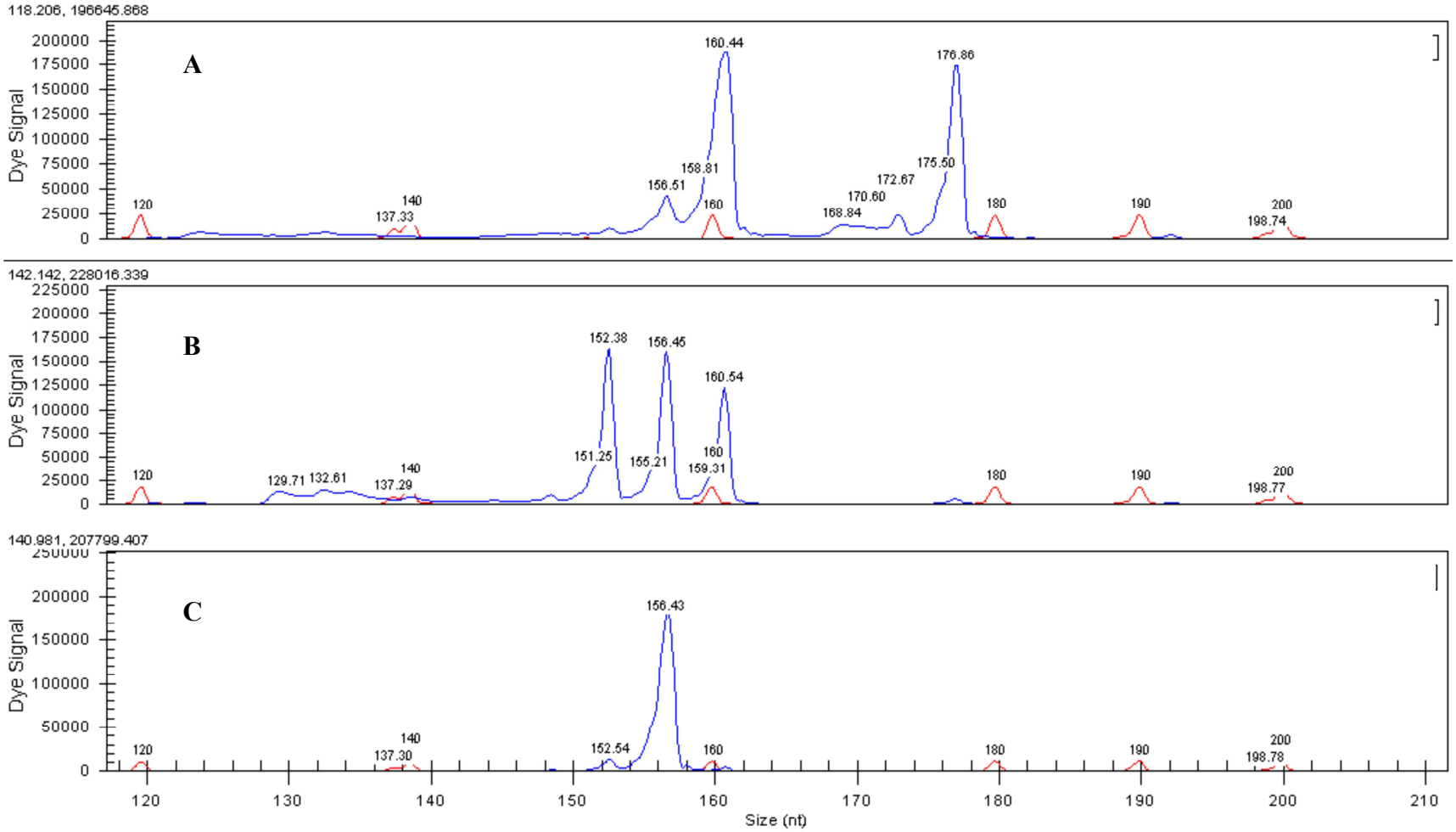
Table 3A. Panel information of brown trout microsatellite loci including, sample number (Trip 1-24), number of alleles (1, 2 or 3 alleles) observed at each locus, and ploidy status of individuals (trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold (- indicates no data observed).

	Fish Identification																							
Panel/Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Ssa410UOS	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	2	**3**	**3**	**3**	2	2	**3**	2	2	**3**
Ssa197	2	2	2	2	2	**3**	2	2	1	2	2	2	2	2	2	2	2	1	2	2	1	1	2	1
ppStr2	2	**3**	2	2	2	**3**	2	-	**3**	2	2	2	2	2	**3**	2	2	2	2	**3**	2	2	2	2
One102-b	2	2	**3**	**3**	2	2	2	-	2	2	**3**	2	3	2	2	**3**	2	2	2	**3**	**3**	2	**3**	**3**
CA048828	2	**3**	**3**	**3**	**3**	**3**	**3**	-	**3**	2	2	2	**3**	2	**3**	**3**	**3**	**3**	2	2	**3**	2	**3**	**3**
ppStr03	2	2	**3**	**3**	**3**	2	**3**	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2
Ssa406UOS	1	2	**3**	2	2	2	2	-	2	**3**	**3**	2	**3**	**3**	2	2	2	2	**3**	**3**	2	**3**	2	**3**
One 103	2	2	**3**	2	**3**	**3**	2	-	2	2	2	2	2	2	2	**3**	1	2	1	2	2	2	2	2
SSa416	1	2	1	1	2	1	2	-	2	1	1	1	1	1	2	1	2	1	1	1	1	2	2	1
SSaD48	**3**	2	**3**	2	2	**3**	**3**	-	**3**	2	2	**3**	**3**	**3**	**3**	**3**	2	**3**	**3**	2	**3**	2	**3**	**3**
Cocl-Lav-4	1	1	1	2	1	2	1	-	**3**	2	2	**3**	**3**	**3**	**3**	**3**	2	**3**	**3**	2	**3**	2	**3**	**3**
One9uASC	**3**	1	1	2	1	1	2	-	2	2	2	2	1	1	1	1	1	2	1	2	2	1	2	2
One102a	1	2	1	1	1	1	1	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA054565	1	1	1	1	1	1	1	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA053293	1	2	2	2	**3**	**3**	2	2	1	**3**	2	1	2	1	**3**	2	2	2	2	**3**	2	2	2	2
CA060177	1	**3**	2	2	2	2	2	-	1	2	2	2	1	1	**3**	2	2	1	2	**3**	1	2	2	1
MHC1	2	2	2	2	2	**3**	2	1	2	2	2	2	1	2	2	2	**3**	2	2	2	2	2	2	2
SSaD71	2	2	2	2	2	2	2	2	2	2	**3**	2	2	2	2	2	2	2	2	2	2	2	2	2
SSaTap2A	2	2	2	**3**	1	2	2	2	1	2	**3**	2	**3**	2	1	2	2	**3**	**3**	2	2	2	2	**3**
BG93548	2	2	2	1	**3**	1	2	2	2	1	2	2	-	-	-	-	-	-	-	-	-	-	-	-
SSa85	1	1	2	1	1	1	1	2	1	2	2	1	1	2	1	2	2	2	2	**3**	2	1	1	2
No. of Loci with 3 alleles	**3**	**4**	**7**	**5**	**6**	**8**	**4**	**1**	**5**	**3**	**5**	**3**	**7**	**4**	**6**	**6**	**3**	**5**	**4**	**6**	**5**	**1**	**4**	**7**
Ploidy status	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip

Table 3B. Panel information of brown trout microsatellite loci including, sample number (Trip 25-48), number of alleles (1, 2 or 3 alleles) observed at each locus, and ploidy status of individuals (trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold (- indicates no data observed).

	Fish Identification																							
Panel/Locus	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Ssa410UOS	**3**	**3**	**3**	**3**	2	2	**3**	**3**	**3**	2	**3**	**3**	**3**	2	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	2
Ssa197	2	2	2	**3**	1	1	2	2	2	**3**	2	2	1	2	1	2	1	2	**3**	2	2	2	2	2
ppStr2	**3**	2	2	**3**	1	**3**	2	**3**	**3**	2	**3**	2	1	2	**3**	2	2	-	**3**	**3**	2	**3**	**3**	2
One102-b	2	2	2	**3**	1	**3**	**3**	2	2	**3**	**3**	2	**3**	2	2	**3**	**3**	-	**3**	2	2	2	2	**3**
CA048828	2	**3**	2	**3**	1	**3**	**3**	**3**	**3**	2	**3**	2	2	**3**	2	**3**	2	-	**3**	2	2	**3**	2	2
ppStr03	2	1	1	2	2	2	2	2	**3**	2	2	1	**3**	2	1	2	2	**3**	**3**	2	2	2	2	2
Ssa406UOS	2	2	2	2	2	**3**	2	2	2	**3**	**3**	**3**	2	**3**	2	2	2	-	**3**	2	1	2	2	2
One 103	1	2	**3**	2	**3**	1	**3**	2	**3**	**3**	**3**	**3**	2	**3**	**3**	2	2	-	**3**	2	**3**	2	2	2
SSa416	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	2	-	1	1	1	1	1	2
SSaD48	**3**	1	2	**3**	2	**3**	**3**	2	2	**3**	2	2	2	**3**	2	**3**	**3**	-	**3**	2	**3**	2	**3**	**3**
Cocl-Lav-4	2	1	1	2	2	1	1	1	1	1	1	1	2	1	1	2	1	-	2	2	1	2	1	1
One9uASC	2	2	1	2	2	2	2	2	1	2	2	2	1	1	2	2	2	-	2	1	2	1	1	2
One102a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-	1	1	1	1	1	1
CA054565	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-	1	1	1	1	1	1
CA053293	2	2	2	**3**	2	1	2	2	2	2	2	2	2	2	1	**3**	**3**	-	**3**	3	2	2	2	2
CA060177	2	1	1	**3**	1	1	2	2	1	1	1	2	1	1	2	2	**3**	1	1	2	2	1	2	1
MHC1	2	2	1	2	2	1	2	2	2	2	2	1	2	1	2	1	2	1	2	2	2	1	1	**3**
SSaD71	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	**3**	2	2	2	2
SSaTap2A	**3**	1	2	**3**	**3**	2	2	1	1	2	1	2	**3**	2	2	2	2	**3**	2	2	1	1	2	2
BG93548	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SSa85	2	1	1	2	1	1	2	1	2	1	2	1	1	1	2	1	1	-	2	2	2	2	1	1
No. of Loci with 3 alleles	**4**	**2**	**2**	**9**	**2**	**5**	**5**	**3**	**5**	**5**	**6**	**3**	**4**	**4**	**3**	**5**	**5**	**3**	**10**	**4**	**3**	**3**	**3**	**3**
Ploidy status	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip

Figure 2. Chromatograph output for brown trout Locus SSa197 and the expected DNA profile of a) two alleles b) three alleles and c) one allele present. Individual fish were only assessed as being triploid if three alleles were present (trisomy) on any given locus.



4. Discussion

The production of triploid brown trout either experimentally or commercially necessarily requires a method of validation. Studies indicated that the methods used so far to verify triploidy in brown trout might not be applicable to commercial screening of triploid brown trout. These techniques are time-and labour-intensive such as karyotype analysis (Arai & Wilkins, 1987; Quillet et al., 1991) or RBC measurements (Crozier & Moffatt, 1989a). In addition, these methods have disadvantages such as the speed of the technique not being suitable for the mass screening of individuals, contain errors by range overlap in cellular and nuclear size between ploidy or require blood for analysis and therefore not applicable to all age groups and therefore cannot provide an early assessment (i.e. RBC measurements). Flow cytometry is an accurate method for assessing triploidy in brown trout (Crozier & Moffatt, 1989a; Preston et al., 2013) however flow cytometry equipment is not routinely available due to high cost associated with the purchase and maintenance of the equipment. In this study, an accurate, cost-effective, fast and non-invasive method using microsatellite markers to verify ploidy status in brown trout at any stage of development was assessed.

Previously, microsatellite markers have been utilised in aquaculture to determine the ploidy status of triploids including abalone *Haliotis midae* (Slabbert et al., 2010), silver crucian carp *Carassius auratus* (Bai et al., 2011), turbot *Scophthalmus maximus* (Hernández-Urcera et al., 2012) and Pacific oyster *Crassostrea gigas* (Nie et al., 2014). However, the present study is the first to demonstrate that ploidy status can be determined in brown trout using a range of polymorphic centromere-distant microsatellite markers. The probability of detecting an individual with trisomy status relied upon scoring a sufficient number of highly polymorphic markers that are also relatively distant from the centromere (to maximise the detection of crossover events). While allele dosage could be inferred from the fragment fluorescence intensity in some instances, it was not sufficiently reliable for routine scoring in this

study. A similarly conservative approach for identifying triploids has been implemented by others (Hernández-Urcera et al., 2012).

At present, farm assessment of triploidy in brown trout is postponed until trout fry are large enough to obtain blood for examination by nuclear measurements. This technique does not therefore allow an early assessment of triploid status and therefore producers have to rear egg batches that may be suboptimal (poor triploid yields) resulting in potential increased production costs. In addition, if the induction process is not 100 % effective, commercial batches of putative triploids will contain diploids, which failed to respond to the pressure treatment. The modern microsatellite markers reported here would alleviate this bottleneck and allow verification of ploidy at an embryonic stage of development (i.e. eyed-ova stage), which is earlier than most other traditional techniques (except karyotype analysis, Crozier & Moffatt, 1989b). This would benefit triploid producers and allow large commercial batches of hydrostatic pressure shocked ova to be screened early, ensuring triploidy status prior to on-growing and sale. Post-sale and stock-out of triploids into freshwater fisheries, these microsatellite markers would allow long-term performance monitoring of commercial triploids by genotyping of fin clips at low cost and without sacrifice (non-invasive).

In contrast, while triploid verification by traditional methods such as cellular and nuclear measurements is inexpensive, this method is labour-intensive and therefore not suitable to the mass screening of commercial brown trout stocks. However, although this technique is effective at resolving ploidy levels, the results of the current study suggest that there is an overlap in size measurements to some degree (nuclear and cell minor axes). Triploid brown trout RBC measurements appeared more pronounced in the major axis (longitudinal) than the minor axis (transversal) for both the nuclear and cellular measurements, suggesting that the shape of the triploid erythrocyte is ellipsoidal (Dorafshan et al. 2008). Increased RBC size of triploids has been reported in other species including, tench *Tinca tinca* (Flajshans, 1997),

turbot (Cal et al. 2005), sea bass *Dicentrarchus labrax* (Peruzzi et al. 2005), short-nose sturgeon *Acipencer brevirostrum* (Beyea et al., 2005), Caspian salmon *Salmo trutta caspius* (Dorafshan et al., 2008), red tilapia *Oreochromis mossambicus* (Pradeep et al., 2011) and rainbow trout *Oncorhynchus mykiss* (Ribeiro et al., 2012). While it is plausible that range overlap between diploids and triploids may exist for each RBC parameter measured, little indication to the extent of the overlap has been suggested. In addition, Benfey et al. (1984) indicated the minor axes of both the cell and the nucleus were poor indicators of ploidy.

The precise determination of ploidy level by the direct method of flow cytometry allows the analysis of several hundred individuals per day (Allen, 1983; Lecommandeur et al., 1994; Piferrer et al., 2009). In this study, the DNA content was determined using a homogenate of muscle tissue from the tail region giving a relative geometric mean and relative peak fluorescence intensity value expressed for each ploidy respectively. Triploid trout had a geometric mean fluorescence, which was 1.5 times greater than diploid trout. Previous studies indicated that ploidy determination by flow cytometry has been achieved in Atlantic cod *Gadus morhua* (Peruzzi et al., 2007; Derayat et al., 2013), Amazon molly *Poecilia formosa* (Lamatsch et al., 2000), tench (Bytyutskyy and Flajshans, 2014) and rainbow trout (Thorgaard et al., 1984). In addition, it has been shown that the fluorescent dye propidium iodide (PI) (Peruzzi et al., 2007) or 4',6-diamidino-2-phenylindole (DAPI) (Bytyutskyy and Flajshans, 2014) are equally effective at binding stoichiometrically to the DNA thus incorporating an amount of dye which is proportional to the amount of DNA. After excitation, the emitted fluorescence signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission of the cell (Nunez, 2001). Brown trout blood was used in this study as internal standard however recent studies indicated that chicken blood *Gallus gallus domesticus* (Peruzzi et al., 2005; Bytyutskyy & Flajshans, 2014, black carp *Mylopharyngodon piceus*, tilapia Jenkins & Thomas, 2007) or rainbow trout blood (Vindelov et al., 2003) are also reliable standards for internal

controls. Flow cytometry is an accurate technique for assessing triploidy however due to the high cost associated with equipment and maintenance, microsatellite markers would appear more applicable to the brown trout aquaculture and fisheries industry. In addition, by refining the current panel of markers, and by utilising six of the most informative microsatellites (Ssa410UOS; One 102-b; CA048828; Ssa406UOS, Ssa410UOS and One 103) would be sufficient to detect triploidy with 100 % accuracy in the current population. This would reduce the number of markers while ensuring a robust screening process. While this work was accomplished using DNA sequencer and fluorescence technology it may be possible to resolve these alleles using standard PCR followed by high resolution agarose electrophoresis, which may be of more practical value to individual hatchery managers.

In this study, a set of highly polymorphic and centromere-distant microsatellites were identified which accurately confirmed ploidy status. Microsatellites may provide aquaculture and freshwater fisheries with the most accurate, economic, rapid, and non-invasive tool over traditional methods for verification of triploidy in brown trout and ensure compliance with stocking legislation.

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CHAPTER 4.

RESEARCH ARTICLE

SURFACE FEEDING AND AGGRESSIVE BEHAVIOUR OF DIPLOID AND TRIPLOID BROWN TROUT *SALMO TRUTTA* DURING ALLOPATRIC PAIRWISE MATCHINGS

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Keywords: Dominance hierarchy, behaviour, aggression, feeding, stream, ploidy.

Abstract

Diploid and triploid brown trout *Salmo trutta* were acclimated for six weeks on two feeding regimes (floating/sinking pellets). Thereafter, aggression and surface feeding response were compared between pairs of all diploid; all triploid and diploid and triploid brown trout *Salmo trutta* in an experimental stream. In each pairwise matching, fish of similar size were placed in allopatry and rank determined by the total number of aggressive interactions recorded. Dominant individuals initiated more aggression than subordinates, spent more time defending a territory and positioned themselves closer to the surface food source (*Gammarus pulex*) whereas subordinates occupied the peripheries. In cross-ploidy trials, diploid trout were more aggressive than triploid, and dominated their conspecific when placed in pairwise matchings. However, surface feeding did not differ statistically between ploidy irrespective of feeding regime. Triploids adopted a sneak feeding strategy while diploids expended more time defending a territory. In addition, this study tested whether triploids exhibit a similar social dominance to diploids when placed in allopatry. Although aggression was lower in triploid pairs than in the diploid/triploid pairs, a dominance hierarchy was also observed between individuals of the same ploidy. Dominant triploid fish were more aggressive and consumed more feed items than subordinate individuals. Subordinate fish displayed a darker colour index than dominant fish suggesting increased stress levels. However, dominant triploid fish seemed more tolerant of subordinate individuals and did not display the same degree of invasive aggression as seen in the diploid/diploid or diploid/triploid matchings. These novel findings suggest that sterile triploid brown trout feed similarly but are less aggressive than diploid trout. Future studies should determine the habitat choice of triploid trout after release and the interaction between wild trout and triploids during the breeding season prior to utilisation of triploids as an alternative management strategy within freshwater fisheries.

1. Introduction

Competition for resources leads to dominance hierarchies (social ranking) forming across a wide range of animal taxa including salmonids (Grant 1993; Kaspersson et al. 2010). Dominance gained through competitive encounters, irrespective of species, leads to a fairly uniform advantage with increased access to food, shelter, and reproductive opportunities (Harwood et al. 2003). Numerous factors can influence social ranking in a dominance hierarchy in vertebrates. In mammals, these have included individual size and number of aggressive acts in domesticated juvenile pigs *Sus domesticus* Erxleben 1777 (Meese & Eubank, 1972), age in American bison *Bison bison* L. 1758 (Robitaille & Prescott 1993), body weight in Mhorr gazelle *Gazella dama* Pallas 1766 (Cassinello & Pieters 2000), aggression in Mountain goat *Oreamnos americanus* Blainville 1816 (Cote 2000) or scent in the Blackbuck *Antelope cervicapra* L. 1758 (Rajagopal 2010). In fish, dominance hierarchies have been reported in many species (e.g. David et al. 2007; McGhee & Travis, 2010; Reddon et al. 2011) and are frequently determined by body size and the number of antagonistic behavioural acts from one individual to another (Sloman & Armstrong 2002).

Many studies in fish have used salmonids to examine the implications of social ranking on foraging success, whereby the rate of aggressiveness has been positively correlated with food intake (Grant 1990; Bryant & Grant, 1995; Adams et al. 1998; Cutts et al. 2001; Harwood, 2002; Höjesjö et al. 2005; Kaspersson et al. 2010). As a result, subordinate individuals may change behaviour and adopt alternative feeding strategies in the presence of dominant fish. Subordinate fish may feed at different times (Harwood et al. 2002), occupy different niches within the same habitat (Kaspersson et al. 2012) or adopt sneak feeding strategies in the presence of dominant individuals to access a limited food resource (Alanärä et al. 1996; Bailey et al. 2000; Höjesjö et al. 2005). Although many studies have examined social dominance hierarchy formation and consequences within members of the same species,

little attention has been given to how intraspecific social dominance hierarchies form and their consequences in individuals with different ploidy levels.

Triploid salmonids, which are sterile and have three sets of chromosomes (3n), are currently produced in the aquaculture and fisheries management sectors as an effective way to prevent unwanted genetic introgression between farmed and wild fish, and to prevent maturation-related effects on important production traits such as growth performance, survival and flesh quality (Taranger et al 2010). However, freshwater sport fisheries are often reliant on supplemental stocking of farmed fish to support catches and increase fishery populations. As a conservation measure the UK Environment Agency (EA) has introduced new legislation to protect wild brown trout *Salmo trutta* L. 1758 populations in England and Wales. By 2015, all trout stocked into all but totally enclosed waters with no significant natural *S. trutta* populations must be either sterile all-female *S. trutta* or from breeding programmes using locally sourced brood fish (EA 2009). Triploid *S. trutta* of farm genetic origin are now being stocked into natural environments to support sport fishery populations to ensure reproductive and genetic containment of farmed fish (EA, 2010). However, with regards to *S. trutta* little is known of the interaction between ploidy post-stockings in wild environments.

Feeding and aggressive behaviour have been well studied in diploid salmonids, including *S. trutta* (Höjesjö et al. 2004, Kaspersson et al. 2010), in allopatric (single species) and sympatric (mixed-species) conditions (Harwood et al. 2002). However, less well understood is whether social hierarchies exist amongst allopatric triploid *S. trutta*. Previous studies have investigated aggression and feeding in communally reared diploid and triploid Atlantic salmon *Salmo salar* L. 1758 (Carter et al. 1994; O'Flynn et al. 1997) and rainbow trout, *Onchorhynchus mykiss* (Walbaum 1792) (Bonnet et al. 1999). These studies indicated that triploids are more prone to fin erosion and suffer reduced growth rates when reared communally with diploid siblings. Aggression trials revealed that triploid Chinook salmon

Oncorhynchus tshawytscha Walbaum 1792 were significantly less aggressive during feeding than diploids in mixed groups (Garner et al. 2008). However, limited knowledge exists on how the surface feeding response is influenced by feeding regime between diploid and triploid *S. trutta* when acclimated to different commercial diets. An evaluation of the surface feeding response and behavioural interactions between ploidy groups is therefore essential, as stocked fish will freely interact with wild conspecifics. The consequences of these behavioural interactions must then be considered within an ecological perspective of freshwater fisheries.

The aim of this study was to compare social aggression, hierarchy and feeding behaviour of diploid and triploid brown trout conspecifics in pair-matched trials in an experimental stream environment. Trials were based on four main hypotheses: (1) social dominance exists within triploid brown trout; (2) aggressive dominant fish would capture more food items than less aggressive subordinates, (3) diploid trout would be more dominant than triploid trout and (4) previous feeding regime (floating/sinking pellet) would influence the surface feeding response of diploid and triploid brown trout post-stocking within the experimental flume.

2. Materials and Methods

2.1 Fish and feed conditioning

A commercial batch of mixed sex diploid (2N) and triploid (3N) diploid *S. trutta* were produced in October 2011 at the University of Stirling's Howietoun Fishery, Stirling, Scotland. *S. trutta* eggs (Loch Leven strain) and milt (Howietoun strain) were collected and pooled into one large batch for fertilisation (n = 25 ♀, n = 12 ♂). The batch was then split into two equal groups, with one group subjected to hydrostatic pressure shock to induced polar body retention (3N), while the other received no shock (2N), giving two groups (diploid (2N) and triploid (3N) ~30,000 / group). Triploids were produced by exposing newly fertilised eggs

to a hydrostatic pressure shock of 69 MPa (690 bar) for 5 minutes, 300 Centigrade Temperature Minutes (CTM) post-fertilisation (Preston et al., 2013). Fish were hatchery-reared in 1m production tanks (ploidy discrete) prior to stocking out into two earthen ponds at ~ 5 grams. Thereafter, yearling *S. trutta* were seine-netted from the two ponds and graded to obtain similar-sized diploid (n=125, mean length L_F 202.3 ± 0.8 mm, weight mean 135.3 ± 5.1 g) and triploid (n=125, mean length L_F 207.7 ± 0.8 mm, mean weight 131.3 ± 5.6 g) groups and transported to the Scottish Centre for Ecology and the Natural Environment, University of Glasgow, Rowardennan, Scotland. Triploid status was subsequently verified by blood smear and erythrocyte nuclei length measurement (mean diploid length: 5.7 ± 0.24 µm, mean triploid length: 8.3 ± 0.21 µm, $P < 0.05$). Fish were randomly assigned into eight 175 L tanks with a flow rate of 10 L / min (ploidy discrete, 4 tanks/ploidy, 30 fish/tank) and acclimated to two different feeding regimes for 6 weeks (May 2012 - June 2012) in order to mimic farm feeding prior to stocking out into an experimental stream. Fish were hand fed to satiation using an industry standard floating or sinking trout pellet (Skretting nutra parr) in duplicate experimental design: (2 feeding regimes: floating/sinking; 2 ploidies: diploid, triploid; total 8 treatments). All fish were placed on simulated natural photoperiod (SNP) using artificial light for this latitude (56°N -18 hours of daylight followed by 6 hours of darkness).

2.2 Pairwise matchings

Three different pairwise fish combinations were investigated: 1- diploid vs. diploid (dip-dip); 2- triploid vs. triploid (trip-trip) and 3- diploid vs. triploid (dip-trip) matchings. In each pairwise combination we placed individuals acclimated to the same feeding regime into pairs and determined the aggression and feeding rate according to the feeding regime given. All fish were randomly selected from the holding tanks and size-matched for length (<10 mm) (Table I). Fish were marked on both lateral and ventral sides with Alcian blue dye (64 mg/mL, Sigma

Aldrich, England) to aid identification within the experimental stream (Bridcut, 1993). Pairs of trout of similar size and on the same feeding regime were placed into the experimental stream, one at a time, until all 10 test arenas were occupied (Fig. 1a).

Table 1. Mass, fork length and Fulton's condition factor (K) of diploid and triploid brown trout measured prior to allopatric pairwise matchings in 1- diploid vs. diploid (dip-dip), 2- triploid vs. triploid (trip-trip), 3- diploid vs. triploid (dip-trip). Superscript lower case letters denote significant differences between treatments ($P < 0.05$). Data presented as Mean ± SEM.

Treatment		
1. Dip-Dip	Dominant diploid	Subordinate diploid
Weight (g)	149.77 ± 11.48[a]	131.64 ± 7.40[a]
Length (mm)	227.20 ± 2.81[a]	225.60 ± 3.53[a]
K factor	1.27 ± 0.10[a]	1.14 ± 0.03[a]
2. Trip-Trip	Dominant triploid	Subordinate triploid
Weight (g)	164.08 ± 7.09[a]	151.64 ± 7.40[a]
Length (mm)	239.00 ± 3.42[a]	236.72 ± 3.05[a]
K factor	1.19 ± 0.02[a]	1.13 ± 0.03[a]
3. Dip-Trip	Diploid	Triploid
Weight (g)	135.27 ± 5.06[a]	131.84 ± 5.65[a]
Length (mm)	219.30 ± 2.47[a]	221.3 ± 2.41[a]
K factor	1.28 ± 0.04[a]	1.20 ± 0.03[a]

The experimental stream was oval (0.6 m wide and 0.6 m deep), with the arms of each side divided into 10 equally sized test arenas (0.9 m in length). The substrate was homogeneously landscaped with gravel (mean diameter: 20.3 ± 5.1 mm) and small pebbles (mean diameter: 45.8 ± 10.3 mm). An electric impellor ensured uniform velocity (0.15 m/s) throughout the flume with water exchange of 15 L per minute (Fig. 1b).

During the feeding trials dried freshwater shrimp *Gammarus pulex* L. 1758 was used to mimic a natural food source commonly found within British freshwater river systems. Dried shrimp was introduced on the surface of the water in the middle upstream position of the test arena, using a polyethylene feed delivery system (Fig. 1b), ensuring each fish had an equal opportunity to capture surface food items. This allowed evaluation of how successful diploid and triploid *S. trutta* were at adapting to a natural food source post stocking into the experimental stream. All fish were acclimated in the experimental stream for 48 hours prior to data collection. The experimental work was reviewed by the University of Stirling Ethics Committee and conducted under UK Home Office licence. No adverse effects were seen on the experimental fish.

2.3 Data collection

Observations consisted of visual recording of aggression, feeding, swimming behaviour and position in each pair during a four minute period. After two minutes, food items (*Gammarus*) were delivered at a rate of one item every 30 seconds (5 feed items per observation period). Three observational periods were monitored each day between 0800-1000, 1300-1500 and 1700-1900 hours for the duration of the experiment (June-August 2012). For the dip-dip treatment, 10 individual pairwise matchings were studied yielding a total of twelve observation periods per pair (3 periods/pair/day) giving a total of 120 observation periods. For the trip-trip and dip-trip pairwise combinations, 20 individual pairwise matchings were observed for each treatment yielding a total of 12 observations per pair (240 observations for each treatment). Dip-dip pair matchings have previously been well documented; therefore, numbers of pairwise matchings were reduced in this treatment.

Agonistic behaviour was defined by the number of aggressive behavioural acts (categorised as splayed fins, mouth gaping, displacement, chases, charges and bites) exhibited

by individual fish on recipients (Adams et al. 1995). Percentage use of each aggression type was calculated by dividing the total of each aggression type by the sum of all the aggressive acts initiated within each pair-wise matching expressed as a percentage. Aggression rate was standardised by dividing the total number of aggressive acts by the total time elapsed giving a rate of aggression per fish per minute. Similarly, for surface feeding responses the total number of prey items consumed was divided by the time elapsed giving a surface feeding rate per fish per minute. Positional index (I_p) was calculated as the proportion of total time spent downstream (3), in the centre (2) or upstream (1) of the arena using the equation: ((*a**100)*3 + (*b**100)*2 + (*c**100)*1) / 100)). Swimming index (I_s) was calculated as the proportion of total time spent at the surface (3), mid-water (2) or bottom (1) of the water column using the equation: ((*a**100)*3 + (*b**100)*2 + (*c**100)*1) / 100)) where *a; b* and *c* is the total time spent within that area of the test arena. (Figure 1b). Body colouration was assessed visually in each pairwise matching on a scale from 1 to 4, with 1 being bright and 4 being dark, of individual fish to indicate potential social stress in subordinate individuals (Keenleyside & Yamamoto 1962; Kaspersson et al. 2010). Fish were removed from the experimental stream once trials had ended and replaced with naive fish from the holding tanks. Dominant fish in each pairwise matching were classified as such when they displayed 60 % or more of aggressive interactions in each pairwise matching (Adams et al. 1995). In three pairwise matchings dominant fish could not be determined; therefore these were excluded from any further data analysis.

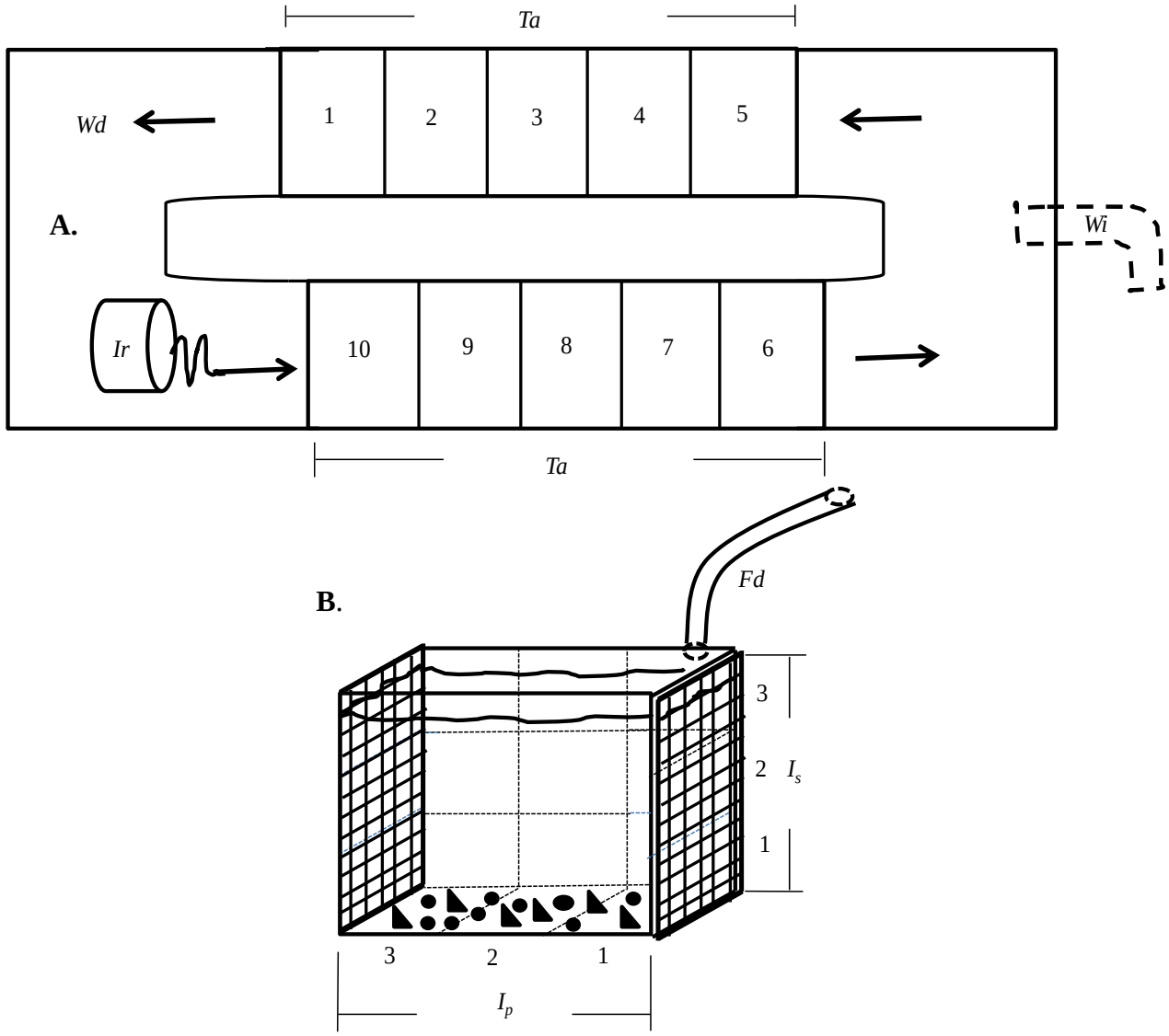

Figure. 1. (a) Schematic view from above the experimental stream with the arms of each side divided into equally sized test arenas (*Ta*) subdivided by mesh grids; arrows indicate water direction (*Wd*), with impellor (*Ir*), and water inflow (*Wi*), (b) An individual test arena showing the homogeneously landscaped substrate with gravel (▲) and small pebbles (●). Dotted lines represent zones (1; 2 and 3) used to calculate the movement of diploid and triploid brown trout between areas within the test arena.

2.4 Statistical Analysis

Statistical analyses were performed using Minitab v15 statistical software (Minitab, Coventry, UK. http://www.minitab.com/en-us/products/minitab/). Normality and homogeneity of data were checked using the Anderson Darling and Levene's test respectively. Due to failure of normality, a Kruskal-Wallis non-parametric ANOVA was used to test aggression rates, feeding rates and the colour index. A Mann-Whitney U test (two-sample Wilcoxon rank sum) was used to test for differences in growth parameters (weight, length and Fulton's condition factor-K), position/swimming index and whether feed regime influenced the surface feed response between dominant fish in each pairwise combination. A similar statistical approach was used by Kaspersson et al. (2010) for detecting differences in feeding, aggression and positional data. All statistical comparisons were performed using a significance level of 5 % ($p < 0.05$). Results are presented as mean ± SEM.

3. Results

3.1 Aggression rates

All known types of aggressive interactions (% of aggressive displays) were observed in the dip-dip treatment and ranked from most to least observed as chase (33.5 %), fin displays (26.5 %), displacement (23.8 %), bite (12.9 %), mouth gaping (2.1 %) and charge (1.2 %) (Fig. 2a). Aggression rates of dominant diploid trout were 1.23 ± 0.55 aggressive acts/min. while no aggression was observed from subordinate diploid trout fed on a floating diet (Fig 3a). Similarly, aggression rate in dominant diploid trout fed on a sinking diet was 0.21 ± 0.04 aggressive acts/min. while none was observed in subordinate fish (Fig 3a). Subordinate diploids did not initiate any antagonistic aggression towards dominant diploids during any trials (Fig. 3a).

In the trip-trip treatment, aggressive interactions were categorised as displacement (dominant trip: 31.5 %; subordinate: 13.64 %), fin displays (dominant: 31.2 %; subordinate: 59.09 %), chase (dominant: 25.3 %; subordinate: 21.21 %), bite (dominant: 5.9 %; subordinate: 1.52 %), mouth gaping (dominant: 5.9 %; subordinate: 4.55 %), and charge (dominant: 0.3 %; subordinate: 0.0 %) (Fig. 2b). Aggression rates of dominant triploid trout (0.31 ± 0.05 aggressions/min.) were significantly higher than those of subordinate triploid trout (0.04 ± 0.02 aggressions/min.) fed on a floating diet (Kruskal-Wallis test: H = 10.67, DF = 1, P = 0.001). Similarly, for triploid trout fed on a sinking diet, aggression rate was significantly higher in dominant trout (0.60 ± 0.15 aggressions/min.) than subordinate trout (0.10 ± 0.04 aggressions/min.) (Kruskal-Wallis test: H = 9.80, DF = 1, P = 0.002) (Fig. 3b).

In the dip-trip treatment, aggressive interactions were categorised as fin displays (dip: 40.9 %; trip: 28.87 %), chase (dip: 20.76 %; trip: 26.80 %), displacement (dip: 19.92 %; trip: 20.10 %), bite (dip: 11.86 %; trip: 8.25 %), mouth gaping (dip: 6.36 %; trip: 15.98 %) and charge (dip: 0.21 %; trip: 0.0 %) (Fig. 2c). Aggression rates of dominant trout (0.78 ± 0.14 aggressions/min.) were significantly higher than those of subordinate trout (0.15 ± 0.05 aggressions/min.) fed on a floating diet (Kruskal-Wallis test: H = 10.60, DF = 1, P = 0.001) (Fig. 3c). Similarly, for trout fed on a sinking diet, aggression rate was significantly higher in dominant fish (0.55 ± 0.10 aggressions/min.) than subordinate trout (0.10 ± 0.06 aggressions/min.) (Kruskal-Wallis test: H = 7.25, DF = 1, P = 0.007) (Fig. 3c). On the other hand, when ploidy was considered aggression rates were significantly higher in diploid (0.68 ± 0.17 and 0.45 ± 0.16 aggressions/min.) than in triploid trout (0.25 ± 0.09 and 0.10 ± 0.06 aggressions/min.) on floating (Kruskal-Wallis test: H = 3.99, DF = 1, P = 0.046) and sinking (Kruskal-Wallis test: H = 5.13, DF = 1, P = 0.023) diets, respectively (Fig. 3d).

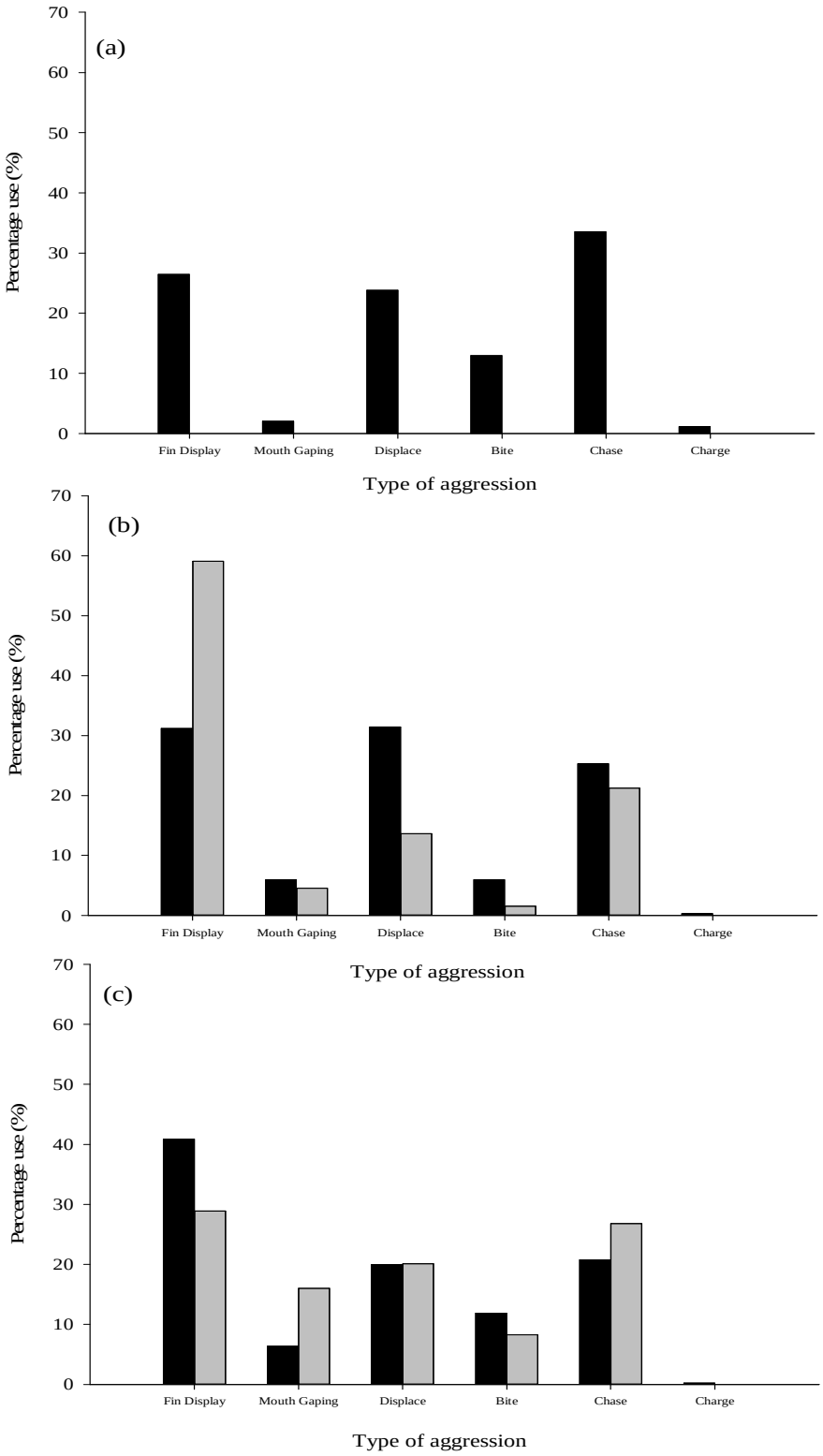

Figure 2. Total aggression (%) expressed as fin displays, displacements, mouth gapes, charges and bites during allopatric pairwise matchings by (a) dominant (■) and subordinate (■) diploid brown trout (dip-dip: n = 120 trials), (b) dominant (■) and subordinate (■) triploid brown trout (trip-trip: n = 240 trials), and (c) diploid (■) and triploid brown trout (■) (dip-trip: n = 240 trials).

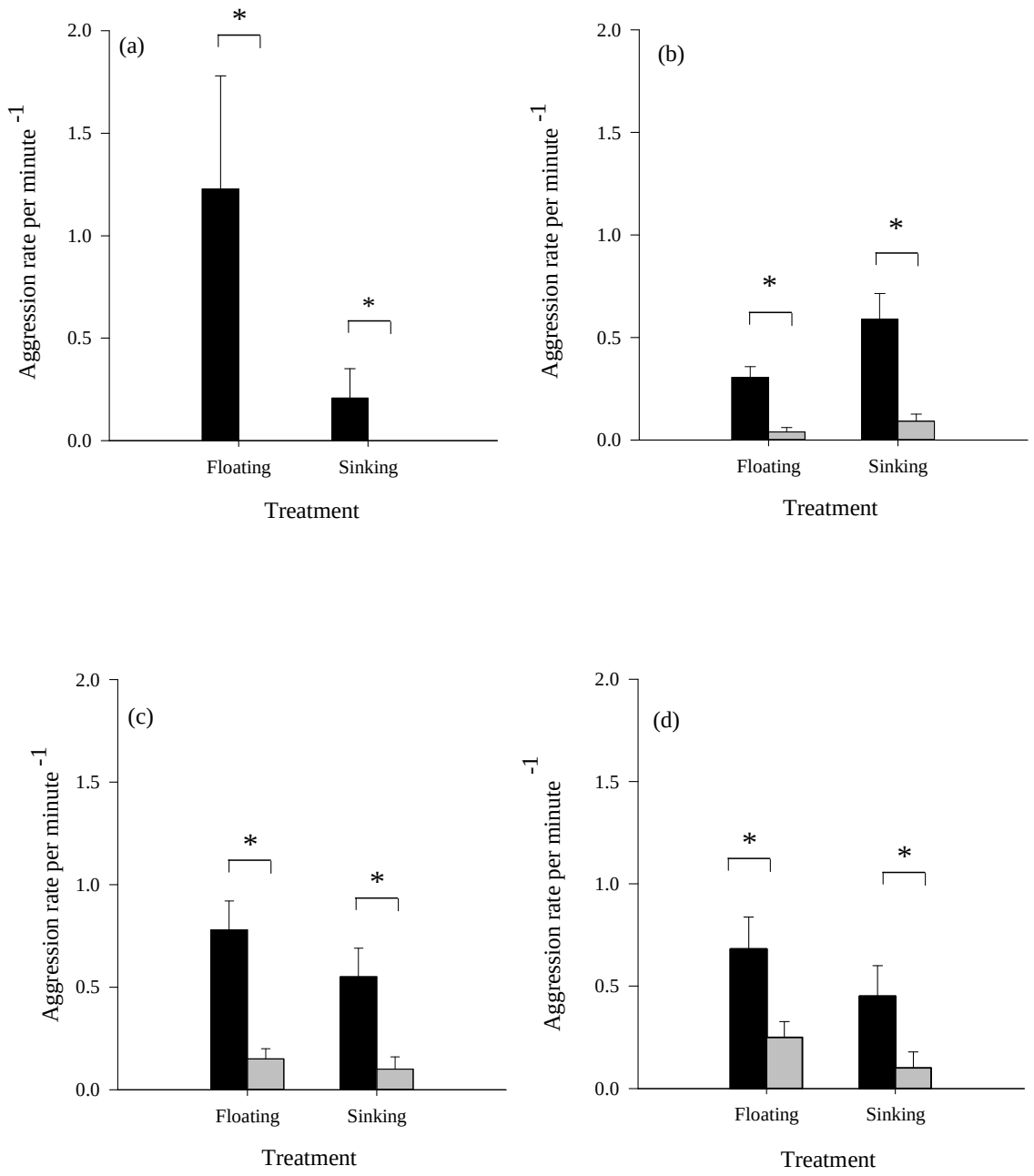

Figure 3. Aggressive acts initiated per minute during allopatric pairwise matchings by (a) dominant (■) and subordinate (■) diploid brown trout (dip-dip: mean ± SEM of 120 trials), (b) dominant (■) and subordinate (■) triploid brown trout (trip-trip: mean ± SEM of 240 trials), (c) dominant (■) and subordinate (■) brown trout (dip-trip: mean ± SEM of 240 trials) and (d) diploid (■) and triploid (■) brown trout (dip-trip: mean ± SEM of 240 trials) previously feed conditioned to a floating and sinking diet respectively. Data are expressed as mean ± SEM. Statistical differences ($P < 0.05$) between pairs are indicated by asterisks (*), ns indicate the lack of significant differences.

3.2 Surface feeding

In the dip-dip treatment, mean number of surface food items consumed per minute differed significantly between dominant and subordinate individuals fed a floating diet (Kruskal-Wallis test: H = 57.65, DF = 1, $P < 0.05$) (Fig. 4a). However, there was no significant differences in the mean number of surface food items consumed when fed a sinking diet (Kruskal-Wallis test: H = 0.02, DF = 1, P = 0.875). Under dip-dip pairings, subordinate individuals failed to consume any feed items during any of the timed trials. On the other hand, dominant trout previously fed a floating diet consumed significantly more surface food items (*Gammarus*) than dominant fish previously fed a sinking diet (Mann-Whitney U Test: W = 80.0, P = 0.012) indicating an effect of feeding conditioning on surface feeding response (Fig. 4a).

In the trip-trip treatment mean food items consumed differed significantly between dominant (1.16 ± 0.30 food items per minute) and subordinate (0.16 ± 0.14 food items per minute) triploid brown trout on a floating diet (Kruskal-Wallis test: H = 5.66, DF = , P = 0.019). In addition, dominant individuals fed a sinking diet consumed significantly more surface food items then subordinates (dominant: 0.65 ± 0.29; subordinate: 0.01 ± 0.01 food items per minute) (Kruskal-Wallis test: H = 5.59, DF = 1, P = 0.018) (Fig. 4b).

In the dip-trip treatment, dominant individuals consumed significantly more food items per minute than subordinates previously fed a floating diet (dominant: 0.90 ± 0.23; subordinate: 0.13 ± 0.09) (Kruskal-Wallis test: H = 6.09, DF = 1, P = 0.014) (Fig 4c). By contrast, mean number of food items consumed by dominant individuals previously fed a sinking diet appeared to be higher than subordinates, however, this was not statistically different (dominant: 0.58 ± 0.28; subordinate: 0.33 ± 0.23) (Kruskal-Wallis test: H = 1.32, DF = 1, P = 0.251). When ploidy (dip-trip) was considered, the mean number of food items consumed was not significantly different between diploid and triploid trout on either floating

(dip: 0.38 ± 0.15; trip: 0.65 ± 0.22) (Kruskal-Wallis test: $H = 0.04$, DF = 1, $P = 0.834$) or sinking diets (dip: 0.44 ± 0.22; trip: 0.47 ± 0.29) (Kruskal-Wallis test: $H = 0.05$, DF = 1, $P = 0.825$) (Fig. 4d).

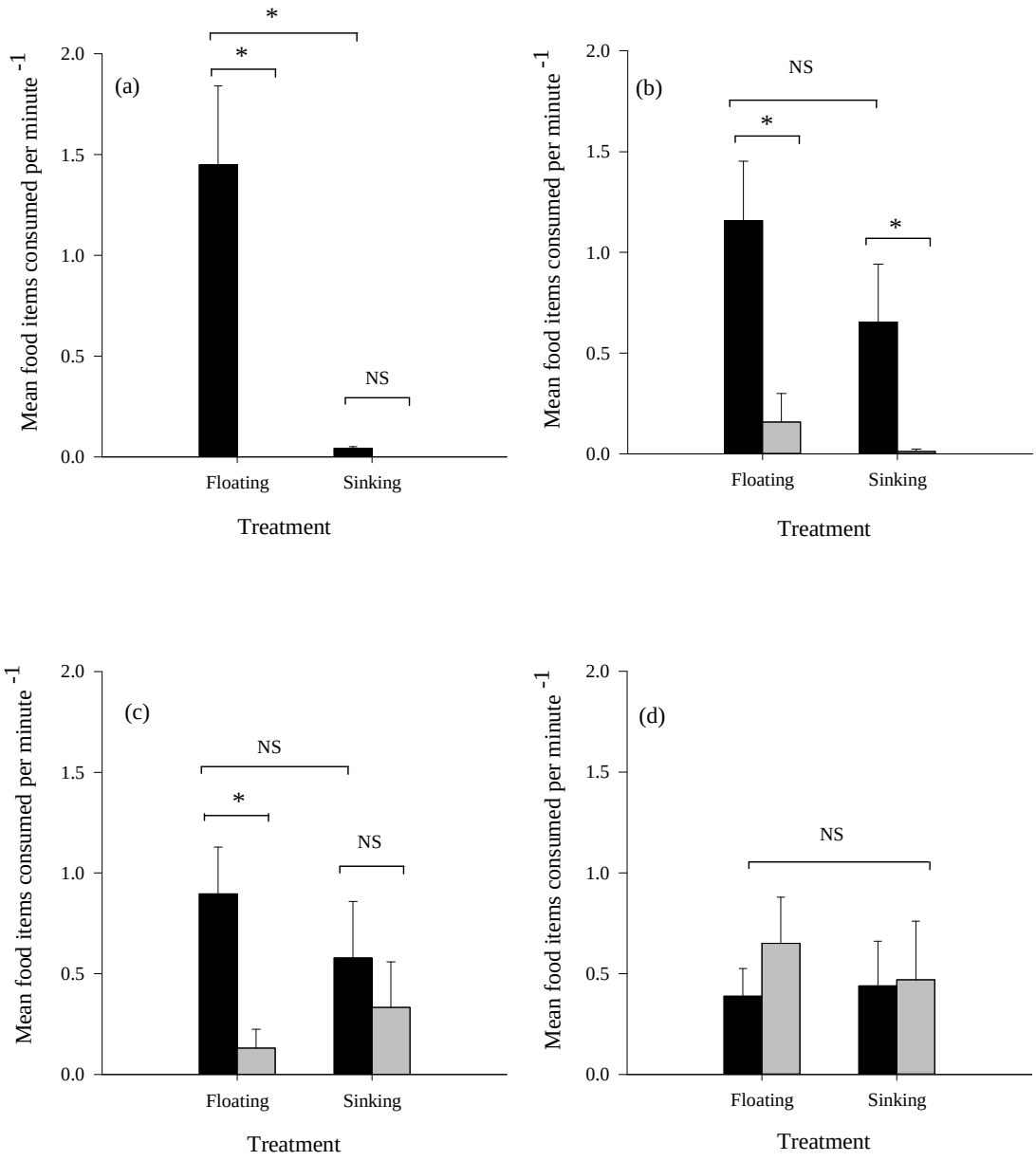

Figure 4. Surface food items consumed per minute by (a) dominant (■) and subordinate (■) diploid brown trout (dip-dip: mean ± SEM of 120 trials), (b) dominant (■) and subordinate (■) triploid brown trout (trip-trip: mean ± SEM of 240 trials) (c) dominant (■) and subordinate (■) brown trout (dip-trip: mean ± SEM of 240 trials) and (d) diploid (■) and triploid (■) brown trout (dip-trip: mean ± SEM of 240 trials) in allopatric pairwise matchings previously feed conditioned to a floating and sinking diet respectively. Statistical differences ($P < 0.05$) between pairs are indicated by asterisks (*), ns indicate the lack of significant differences.

3.3 Swimming and positioning

No statistical differences in the swimming index were observed in the dip-dip, trip-trip and dip-trip treatments (Fig 5a, b, c and d). However, differences in the positional index were observed between treatments. In the dip-dip treatment, dominant fish adopted a more central position (mean = 2.24 ± 0.08) within the experimental flume, which was significantly different to subordinate fish, which tended to occupy a position closer to the rear of the arena (mean = 2.83 ± 0.10) (Mann-Whitney U Test: $W = 66.0$, $P = 0.003$) (Fig. 5a).

In the trip-trip treatment, dominant trout adopted a central position (mean = 2.05 ± 0.09) within the experimental flume, which was significantly different to subordinate (mean = 2.90 ± 0.04) siblings (Mann-Whitney U Test: $W = 158.0$, $P < 0.05$) (Fig. 5b).

In the dip-trip treatment, dominant fish adopted a more central position (mean = 2.26 ± 0.11) within the experimental flume, which was significantly different to subordinate fish (mean = 2.55 ± 0.14) (Mann-Whitney U Test: $W = 233.0$, $P = 0.027$) (Fig. 5c). When ploidy was considered within the same treatment diploid fish adopted a similar position to triploid siblings (mean diploid = 2.44 ± 0.11; mean triploid = 2.43 ± 0.15) within the experimental flume (Mann-Whitney U Test: $W = 304.5$, $P = 0.822$) (Fig. 5d).

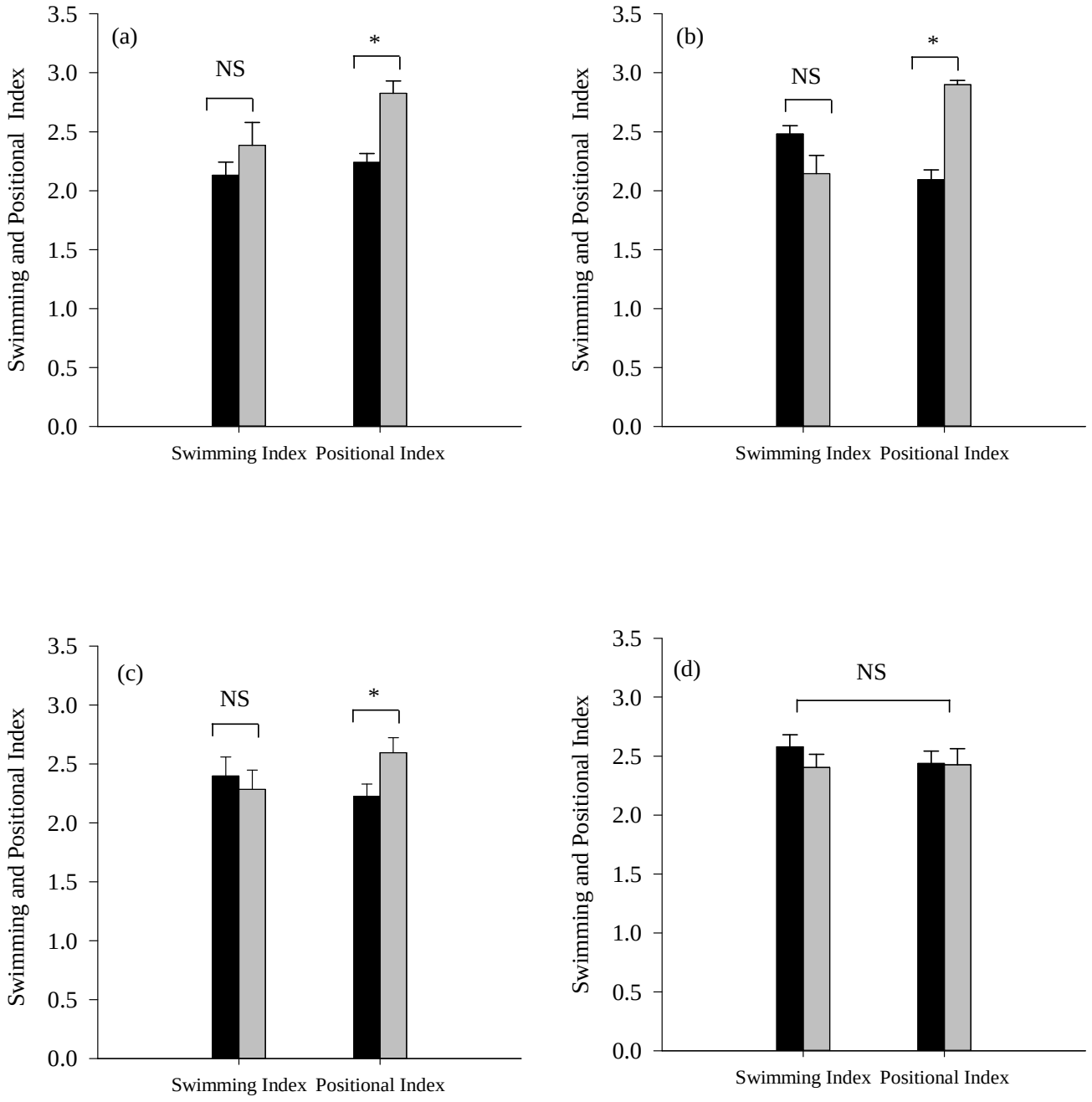

Figure 5. Swimming activity (1: near stationary on the substratum, 2: swimming midwater, 3: near surface) and position index (1: upstream, 2: middle of stream, 3: downstream) for (a) dominant (■) and subordinate (■) diploid brown trout (dip-dip: mean ± SEM of 120 trials), (b) dominant (■) and subordinate (■) triploid brown trout (trip-trip: mean ± SEM of 240 trials), (c) dominant (■) and subordinate (■) brown trout (dip-trip: mean ± SEM of 240 trials) and (d) diploid (■) and triploid (■) brown trout (dip-trip: mean ± SEM of 240 trials). Statistical differences ($P < 0.05$) between pairs are indicated by asterisks (*), ns indicate the lack of significant differences.

3.4 Colour index

Significant differences in mean colour index were found between dominant and subordinate individuals in the dip-dip floating (Kruskal-Wallis test: $H = 6.82$, DF = 1, $P = 0.009$), sinking (Kruskal-Wallis test: $H = 6.82$, DF = 1, $P = 0.009$); trip-trip floating (Kruskal-Wallis test: $H = 10.11$, DF = 1, $P = 0.001$); dip-trip floating (Kruskal-Wallis test: $H = 9.93$, DF = 1, $P = 0.002$) and sinking (Kruskal-Wallis test: $H = 7.02$, DF = 1, $P = 0.008$) (Fig. 6a, b, and c). When ploidy was considered in the dip-trip treatment significant differences were found between diploid and triploid individuals in the floating (Kruskal-Wallis test: $H = 4.19$, DF = 1, $P = 0.041$), and sinking (Kruskal-Wallis test: $H = 4.19$, DF = 1, $P = 0.041$) treatments respectively (Fig. 6d). However no significant difference was observed in the trip-trip sinking (Kruskal-Wallis test: $H = 0.15$, DF = 1, $P = 0.701$) treatment (Fig. 6b).

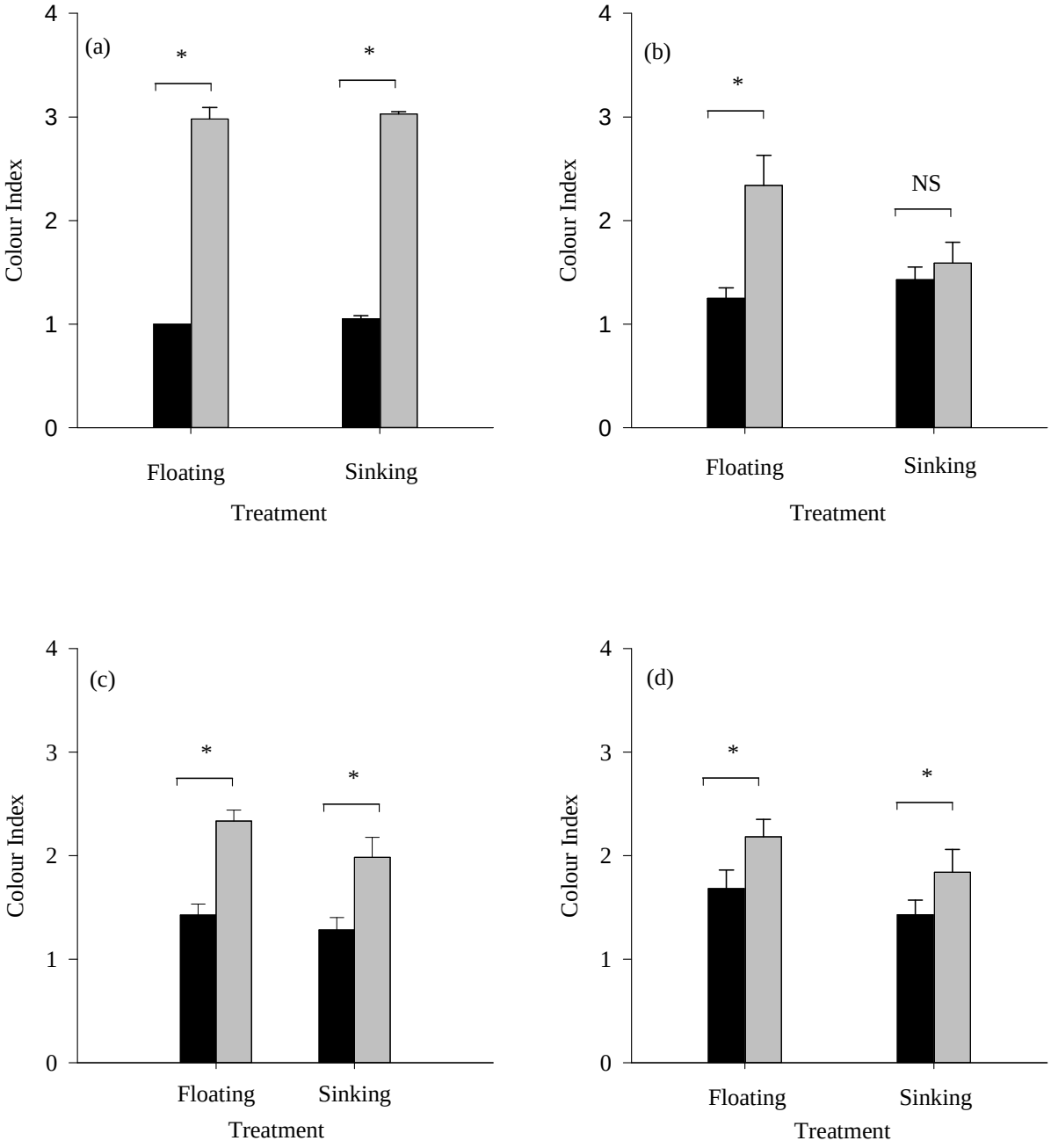

Figure 6. Colour index for (a) dominant (■) and subordinate (▩) diploid trout (dip-dip: mean ± SEM of 120 trials), (b) dominant (■) and subordinate (▩) triploid brown trout (trip-trip: mean ± SEM of 240 trials), (c) dominant (■) and subordinate (▩) brown trout (dip-trip: mean ± SEM of 240 trials) and (d) diploid (■) and triploid (▩) brown trout in allopatric pairs (dip-trip: mean ± SEM of 240 trials). Body colouration was assessed visually in each pairwise matching on a scale from 1 to 4, with 1 being bright and 4 being dark. Statistical differences ($P < 0.05$) between pairs are indicated by asterisks (*).

4. Discussion

Social hierarchies in salmonids are formed and maintained by aggressive interactions between individuals with dominant fish having a higher frequency of aggressive acts towards subordinates (Sloman and Armstrong, 2002). Recent studies indicated that *S. trutta* form hierarchies where dominant individuals monopolise food sources (Höjesjö et al. 2004, Kaspersson et al. 2010). Similarly, in this study the existence of dominance hierarchies in diploid *S. trutta* where dominant individuals in the dip-dip treatment monopolise the entire food source have been demonstrated. Aggression has thus been shown to be a suitable indicator of dominance in salmonids (Metcalfe et al. 1989; Bailey et al. 2000; Tiira et al. 2009; Kaspersson et al. 2010). In the present study, dominance was based on the frequency of overt aggression displayed as fin displays; displacements; charges; chases and nips, which has previously been described (Mesa 1991; Deverill et al. 1999; Cutts et al. 2001).

Although the aggressive behaviour of triploid *S. trutta* is not well characterised, the literature suggests triploid fish have a different behaviour to diploids. Both triploid *S. salar* and *O. tshawytscha* were less aggressive than diploids and exhibited more severe fin damage than diploids when communally reared (Carter et al. 1994; Garner et al. 2008). This has also been observed in non-salmonids where triploid Siamese fighting fish *Betta splendens* Regan 1910 showed less aggressive behaviour (erection of fins or opercula, air gulping, undulating movements, striking and biting) than diploids (Kavumpurath & Pandian 1992). Similar observations were made during this study, where triploid fish appeared to exhibit less fin damage than diploids during the feed conditioning period when ploidy were reared discretely (*personal obs*.). However, aggression does not always differ between ploidy as demonstrated in size-matched pairings of *O. mykiss* (Wagner et al., 2006).

This study is the first report of dominance hierarchies in triploid *S. trutta* within the scientific literature. Results of the trip-trip treatment suggest that the formation of dominance

hierarchies is not influenced by ploidy and that dominance confers an advantage in foraging for dominant triploids as it does for dominant diploid *S. trutta*. These results provide some support for our first assumption that triploid *S. trutta* would display social dominance hierarchies similar to those demonstrated in diploid *S. trutta*, as in this study (dip vs. dip) and in other studies (Kaspersson et al. 2010). On the other hand, the interaction between ploidy during the dip-trip treatment, suggests a similar but asymmetric dominance hierarchy forms, with diploid trout more likely to dominate. These results indicate that diploid *S. trutta* have a high capacity to dominate triploids when placed in allopatry. However, dominance of diploid over triploid fish did not translate into differential resource acquisition as evident in the dip-dip treatment with dominant individuals gaining access to all the food resource. This suggests that during a diploid-triploid interaction, dominance does not confer preferential access to food, indicating that triploid *S. trutta* use a different mechanism to access food resources if in a subordinate position within a dominance hierarchy. Such a feeding strategy was not observed in subordinate diploid trout.

Alternative "sneak" feeding strategies have been described in *S. salar* parr in the presence of dominant *S. trutta* (Harwood et al. 2002) in which subordinate fish adopt a less aggressive strategy and continue feeding. In our study, diploid trout spent more time defending a territory and therefore triploid fish could sneak feed using the peripheries of the test arena. Triploid trout displayed a behavioural plasticity and appeared to adapt their feeding behaviour to their social environment within the experimental stream. This strategy could be beneficial to triploid trout within a wild environment as flexibility in feeding behaviour (coping strategy) could allow monopolization of alternative food sources using a less aggressive strategy in the presence of diploid fish. In *S. salar*, dominant individuals compete aggressively when placed in groups, while less aggressive individuals adopt such alternative feeding strategies, which reduces the risks of injury to subordinates although it results in

reduced feed intake and growth (MacLean et al. 2000). It seems social dominance between diploid and triploid trout, which have similar ecological requirements, promotes the expression of an alternative behavioural strategy in triploid trout as previously described in other allopatric trials (Harwood et al. 2002).

The *S. trutta* used during our study were from a hatchery and therefore a comparison between hatchery and wild fish was not possible. An evaluation of the interactions between hatchery triploid and wild *S. trutta* should be made in light of new legislation (EA, 2009). Triploid trout will be freely interacting with wild *S. trutta* and therefore the ecological interactions between ploidies should be considered. Although the results of such pairwise interaction have not yet been demonstrated experimentally, Deverill et al. (1999) indicated that hatchery diploid *S. trutta* were more aggressive than wild *S. trutta*. Although triploid *S. trutta* appeared less aggressive than similar sized diploid trout in allopatric pairwise matchings, it is not known whether this ecological advantage is extended to wild *S. trutta* trout. In other salmonids, it has been demonstrated that hatchery cutthroat trout *Oncorhynchus clarki clarki* Richardson 1836 can be more aggressive than wild (Mesa 1991), or less aggressive in Chinook salmon *O. tshawytscha* (Pearsons et al. 2007), however, this may depend on experimental conditions. Therefore, further investigation is needed to quantify whether larger triploid size will result in social dominance over wild *S. trutta*.

In the present study, flexibility in the feeding behaviour of triploid *S. trutta* provides a comparative model for studying coping behaviours in salmonids. In a given situation, proactive individuals show active avoidance, more aggression, higher general activity and a predominant sympathetic reaction. On the other hand, reactive individuals respond more with immobility; freezing behaviour and a predominant parasympathetic/hypothalamic activation (Koolhaas et al. 1999). Recent studies indicated the existence of these two distinct coping strategies in *S. trutta* and that trout from different origins differ in the endocrine stress

response and behaviour during hypoxia and aggression (Brelin et al. 2005; Brelin et al. 2008). Previously the existence of proactive and reactive animals has been described as a balance of different traits preserving genes for high aggression (Hawks) and low aggression (Doves) within a population (Korte et al. 2005; Ruiz-Gomez et al. 2008). In the present study, we have shown that diploid trout exhibit higher aggression rates than triploid *S. trutta* suggesting the existence of proactive (diploid) and reactive coping styles (triploid) during diploid and triploid allopatric pairwise matchings. In addition, this study is the first report to suggest these differential strategies within triploids. As in other vertebrates, corticosteroids (cortisol) are the main indicator of stress in fish, and increased blood concentration of cortisol arises from activation of the hypothalamus-pituitary-interrenal (HPI axis) (Wendelaar-Bonga, 1997). Selected lines of *O. mykiss* have been shown to differ in stress responsiveness, with lower cortisol responders (proactive individuals) exhibiting higher growth rates compared to high cortisol responders (Pottinger 2006; Trenzado et al. 2006). In the present study, we observed a consistently darker body colouration of subordinate individuals in each pairwise matching. Colour patterns have been associated with social status and aggressive interactions in juvenile salmonids (O'Connor et al. 1999; Suter & Huntingford, 2002; Kaspersson et al. 2010). Dominant fish in our study exhibited a lighter colour index than subordinates, with subordinates signalling defeat through a darkening in coloration. Darkening of subordinate salmonids is primarily a result of increased stress levels, and acts secondarily in communication to reduce further attacks from dominant individuals (Eaton and Sloman 2011). In our study, no analysis of stress response through cortisol analyses could be done. However, in *O. mykiss* it has been shown that the behaviour of dominant and subordinate individuals was correlated to the magnitude of blood cortisol levels with subordinate fish having increased cortisol levels (Pottinger & Carrick 2001). The effect of subordinate social status has been shown to negatively impact digestive function, metabolism and enzyme activity in

O. mykiss (DiBattista et al. 2006), suggesting that subordinate trout in our study may equally be disadvantaged in the presence of dominant individuals, although such effects are still to be elucidated in *S. trutta*.

The underlying mechanisms for the altered behaviour in triploid fish are not known, however it is possible that the reduction in cell numbers of the central nervous system (CNS) and the reduced levels of endocrine hormones may in part be responsible (Benfey 1999). In diploid fish, increased levels of androgens (testosterone and 11-ketotestosterone) have been linked to territorial dominance and aggression in male African cichlid fish *Haplochromis burtoni* Gunther 1894 (Francis et al. 1992). In contrast, female triploid fish have been shown to have lower levels of sex steroids than diploids (Piferrer et al., 2009), and thus it is possible that reduced circulating endocrine hormones may make triploids less aggressive than diploids. On the other hand, alterations in aggression and feeding ability may relate to cognitive differences due to changes in brain morphology (smaller olfactory bulb, larger cerebellum and telencephalon) reported in triploid *S. salar* (Fraser et al., 2012). Given that size of the cerebellum and telencephalon are linked to aggression, foraging strategy and swimming ability (McIntyre & Healy, 1979; Kolm et al., 2009), it could be expected that triploids would be more aggressive and effective feeders. However, evidence to date including the current study suggests the opposite (O'Keefe & Benfey 1997; Czesny et al. 2002; Garner et al. 2008), and therefore reduced foraging ability may be due to other ploidy-dependent differences. Fraser et al. (2012) also proposed that the increase in size of the triploid cerebellum and telencephalon may not necessarily imply a greater cognitive ability and aggression, as cell numbers are still likely to be less than in diploids, with subsequent effect on neuronal connections.

This study did however, show the association between aggressiveness and increased food intake whereby dominant trout, irrespective of ploidy, consumed more surface food

items than subordinate individuals, thus providing support for our second hypothesis. This is in agreement with the overwhelming evidence linking aggression and increased food acquisition (Grant 1990; Bryant & Grant 1995; Adams et al. 1998; Cutts et al. 2001; Harwood 2002; Höjesjö et al. 2005; Kaspersson et al. 2010). In the dip-dip treatment dominant trout consumed more food items, with subordinates not able to monopolise any food items in either the floating or sinking treatments. Furthermore, it is known that trout in allopatry can adjust methods of resource acquisition based on relative costs and benefits (Harwood et al. 2002). In the present study, diploid trout appear to experience a cost of reduced surface feeding as a result of increased resource defence when in allopatry with triploid trout (dip-trip). Aggressive chinook salmon were shown to displace non-native brook trout *Savelinus fontinalis* Mitchill 1814, however, feeding activity of *O. tshawytscha* declined as encounter rate increased, indicating a cost of resource defence (MacNeale et al. 2010). Similarly our results showed that aggression rates were significantly higher in diploid fish, however, the number of surface feed items consumed was not significantly different between diploid and triploid trout. This suggests diploids incur a cost associated with the increased aggression during allopatric pairwise matching with triploids. High social dominance has been shown to result in substantial costs associated with aggression in terms of increased energy expended during territorial defence, and loss of feeding opportunity during the defence of territory (Adams et el. 1998; Harwood et al. 2002; Harwood et al. 2003). However, the cost of dominance is generally outweighed by the benefits of the resource defence, which is increased food intake (Harwood et al. 2002). Nonetheless, from the current data it does not appear to be the case when diploid and triploid *S. trutta* were in allopatry, as diploids did not consume more feed items. In addition, our results suggest an effect of feeding regime on surface feeding response, whereby dominant individuals previously fed on a floating diet consumed more surface food items than dominant individuals fed a sinking diet. These observations indicate that feed

conditioning to a specific diet during culture may influence the feeding behaviour of brown trout when stocked into freshwater fisheries.

Finally, in this study, dominant fish (positional index ~2) in all three treatments (dip-dip, dip-trip, and trip-trip) occupied a more central position closer to the food source when compared to subordinates. This clearly confers an advantage to exploit the available food resource. Similar results have been shown in sympatric pairwise matching between dominant *S. trutta* and subordinate *S. salar* (Höjesjö et al. 2005). However, in our study, swimming index was not very informative when comparing dominant and subordinate individuals within the experimental stream. Other studies showed differences in swimming activity (movements between individuals within the water column) whereby dominant trout were more active and swimming centrally whereas submissive salmon were displaced to marginal areas (Höjesjö et al. 2005).

This study provides first evidence of dominance hierarchies in both diploid and triploid *S. trutta* with direct implications for management strategy within the aquaculture and fisheries sectors. Diploid trout exhibit higher aggression rates than triploid *S. trutta* confirming the existence of proactive and reactive coping styles during allopatric pairwise matchings. Similarly, triploid *S. trutta* also exhibited a social dominance hierarchy; however, they adopted an alternative "sneak" feeding strategy in the presence of more dominant diploid *S. trutta*. These behavioural interactions between and within ploidy have important implications for freshwater fisheries and the conservation of wild salmonids. Further work is required to establish whether size, gender and reproductive status influence dominance between ploidy within a semi natural environment. Behavioural differences between genders should be established, as the new legislation is for the stocking of all female triploids into freshwater ecosystems.

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CHAPTER 5.

RESEARCH ARTICLE

THE EFFECT OF TEMPERATURE AND PLOIDY ON FEED INTAKE, BLOOD HOMEOSTASIS AFTER EXHAUSTIVE EXERCISE AND VERTEBRAL DEFORMITIES ON DIPLOID AND TRIPLOID BROWN TROUT (*Salmo trutta* L.).

Submitted to: Comparative Biochemistry and Physiology Part A: *Molecular & Integrative Physiology.*

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Contributions: The present manuscript was written and compiled in full by the author of this thesis. Sampling, lab and statistical analysis was carried out by the candidate with the support of thesis supervisors (Professors' Herve Migaud, Per Gunnar Fjelldal, Tom Hansen and Dr John F Taylor) who also proof read the manuscript.

Keywords: simulated capture, thermal, ploidy, exercise, lactate, deformity.

Abstract

This study examined the effects of temperature on feed intake and haematological homeostasis after exhaustive swimming. Diploid and triploid brown trout *Salmo trutta* (~75 g) were exposed to an incremental temperature challenge (2 °C/day) from ambient (6 °C) to 10 °C and 19 °C, followed by a period with stable temperatures (10 °C: 22 days, 19 °C: 18 days). Results indicate similar feed intake profiles at 10 °C however; triploids had a significantly higher feed intake at 19 °C. After 24 days, each temperature-ploidy group was exposed to exhaustive swimming for 10 minutes during a simulated catch-and-release protocol. The fish were screened for spinal deformities by radiology after the swimming test, in order to uncover possible bias effects of deformities on swimming ability. The haematological response was measured before, and then 1 h, 4 h, and 24 h after exhaustion; the response differed between ploidy, with the magnitude of the response affected by temperature and ploidy, despite equal deformity prevalence. Post-exercise triploids had significantly higher osmolality and cholesterol values than diploids, but these returned to resting levels at 24 h post-exercise. The present results may suggest that triploids have greater appetite than diploids at high temperature, and that triploids may be more vulnerable to negative effects of catch and release than diploids. This could have implications for the freshwater sport fisheries sector, which endorse both stocking of triploid brown trout into natural waters with different thermal profiles, and catch and release fishing practices.

1. Introduction

The induction of triploidy has become a popular tool for the production of sterile fish in the aquaculture and fisheries sectors. Triploids have been commercially or experimentally produced in many fish and shellfish species by hydrostatic pressure, heat, cold or chemical shocks (Piferrer et al., 2009). Recently, the benefits of triploidy have become attractive to alleviate potential genetic introgression between farmed and wild fish stocks (Taranger et al. 2010) and also for increased growth rates during the production cycle (Taylor et al., 2013). Currently, farmed all-female triploid (AF3N) brown trout (*Salmo trutta*) are being stocked into recreational freshwater fisheries within England and Wales as part of a National Trout and Grayling Strategy to reduce the risk of breeding with wild brown trout populations (EA, 2009). Triploid trout are cultured using commercial aquaculture practices and then released into freshwater ecosystems to support recreational fisheries. Many fisheries endorse catch and release policies and therefore triploid fish must be able to recover physiologically from strenuous exercise at a variety of temperatures (Clark et al. 2012). However, concerns have been raised for the use of triploids in wild rivers due to their increased sensitivity to fluctuating environmental conditions and exercise tolerance as part of catch and release fisheries (Hyndman et al. 2003, Maxime, 2008).

Triploids have larger nuclei to facilitate the increase of genomic content, with subsequent increases in cellular volume and cell size as well as an overall reduction in cell numbers (Benfey, 1999). The increase in cell size may cause reductions in the cellular surface to volume ratio, which could alter transport processes across cell membranes (Maxime, 2008). In addition, exhaustive exercise imposed by angling capture has been shown to impact on the ion, acid-base and metabolic status of teleosts resulting in significant metabolic acidosis (Wood et al.1983; Lowe and Wells, 1996; Meka and McCormick, 2005; Wells and Dunfy, 2009). Previously, studies have found that the thermal tolerance of triploids is similar to

diploids, as indicated by the time taken to reach critical thermal maxima (CTM) of brook trout *Salvelinus fontinalis* and rainbow trout *Oncorhynchus mykiss* (Benfey et al., 1997; Galbreath et al., 2006). On the other hand, metabolic rates differ between diploid and triploid brook charr and Atlantic salmon (*Salmo salar*), with triploids having higher metabolic rates than diploids at lower temperatures, and lower metabolic rates than diploids at higher temperatures (Atkins and Benfey, 2008).

The thermal tolerance of triploid fish has attracted interest from scientists as increased mortality has been demonstrated at high temperatures (Ojolick et al., 1995). In triploid brown trout, increased mortalities (50%) were observed when reared at 18 °C for 12 weeks (Altimiras et al., 2002). Similarly, Hyndman et al. (2003b) found that diploid brook trout recovered from exhaustive exercise at 19 °C whereas 90 % of triploids died within 4 hours of exhaustive exercise at this temperature. It was suggested that the magnitude of the physiological disturbance was greater in triploid trout, which resulted in failure to restore muscle metabolites back to pre-exercise conditions causing significant mortality. Water temperature may also affect cardiac response, and differences in heart morphology between ploidy have been observed (Fraser et al., 2013). In rainbow trout, heart rate was similar between ploidy at 10 °C, however, at higher temperatures more triploid trout suffered from cardiac disruption than diploids (Verhille et al., 2013). This suggests the reduced tolerance to high temperature in triploid rainbow trout may be due to reduced cardiovascular scope and oxygen delivery (Verhille et al., 2013). Furthermore, triploid fish are also reported to be more prone to skeletal deformities (Leclercq et al., 2011), and it has been shown that spinal malformation affects swimming capacity and recovery in triploid Atlantic salmon (Powell et al., 2009). In this respect it is important to establish if the same is true in brown trout.

The objectives of the present study were (i) to determine the effects of acute temperature increase on feed intake, (ii) investigate the storage and utilisation of plasma metabolites

before and after exhaustive exercise (simulated catch-and-release scenario), and (iii) determine if deformity prevalence differed between ploidy. Differences in plasma haematology post-exercise were used in this study as a biomarker for relative fitness between ploidy. The starting hypothesis was that triploid brown trout would show lower feed intake and slower recovery from exhaustive exercise and exhibit higher levels of deformity than diploids.

2. Materials and Methods

2.1. Fish stock and rearing conditions

On 10th November 2011, eggs from eight female brown trout were pooled into one batch and fertilized with pooled milt from three males at the Institute of Marine Research, Matre, Norway. After fertilization, eggs were split into two batches and one batch of fertilised eggs subjected to a hydrostatic pressure shock of 9500 psi (655 bar) for 6 min and 15 s, 37 minutes and 30 sec post fertilisation at 8 °C (TRC-APV, Aqua Pressure Vessel, TRC Hydraulics inc., Dieppe, Canada), giving two groups (diploid and triploid ~3500 / ploidy). The ova and sperm were obtained from a commercial production site for portion-size brown trout in Tyssedal, Norway. The trout originated from Tunhovd Lake in Eastern Norway, and had been held in culture for ten generations. Each ploidy group was incubated separately in a single incubation tray using a flow-through system at 7 °C. Eggs were mechanically agitated to allow dead eggs to be sorted from live eggs at the eyed-egg stage (~200 ˚days). On 29th February 2012, first-feeding fry from each incubator were randomly distributed between three square grey covered fiberglass tanks (1×1×0.5 m) under continuous light (LL) and at 10 °C (total 6 tanks; 3 per ploidy; 400 fish in each tank). Ambient water temperature was increased from 10 °C to 13.5 °C on 28th August 2012. On the 28th September 2012, numbers of fish in each tank were reduced to 300 in order to reduce stocking density. Thereafter, water temperature decreased

gradually to 6 ^{o}C on 11th December 2012 and remained stable in the range of 5.9 to 6.4 ^{o} C until the 15th January 2013 and the commencement of the feed intake study.

2.2. Feed Intake Study

The fish were reared in 6 tanks (3 replicates / ploidy; 300 fish / tank) until 11th December 2012, when all fish from each ploidy were measured for length and weight, then 320 fish per ploidy were randomly distributed in quadruplicate design among sixteen freshwater tanks with 40 fish / tank (1 x 1 x 0.6m, 0.6 m^{3}), giving eight tanks with diploids (mean weight 70.53 ± 1.02 g; length 164.94 ± 0.74 mm) and eight tanks with triploid (mean weight 79.87 ± 1.18 g; length 173.72 ± 0.80 mm) brown trout. Fish were allowed to acclimate for 48 h before being subjected to a thermal challenge where temperature was increased from ambient (6.5 ± 0.5 °C) to 10 ± 0.8 °C or 19 ± 0.9 °C respectively, in 2° C day^{-1} increments, producing two temperature regimes (10 or 19 ^{o} C by two ploidy: diploid and triploid with 4 replicates each). All tanks were fed three meals per day using ArvoTec 2000 drum feeders and a commercially available 3 mm dry feed (Nutra parr, Skretting, Norway) for a period of 24 days. Each equally sized meal (100 g of dry pellets) was placed in the ArvoTec prior to meal times and dispensed into the tanks over a one hour period from 08.00 to 09.00 (meal 1), 11.30 to 12.30 (meal 2) and 14.00 to 15.00 (meal 3). Waste feed was collected 25 mins after each meal, excess water was drained off and weighed according to the method described by Helland et al. (1996) and used to calculate the dry weight of feed eaten (hereafter called feed intake). Feed intake was calculated relative to the estimated biomass in each tank (% of biomass). The efficiency of the feed collectors was tested in tanks prior to the experiment. A recovery coefficient (RC) was calculated using the mean of 12 tests runs in tanks without fish using the formula: RC =W/A, were W is the wet weight (g) of the waste feed collected and A is the dry weight (g) of pellets supplied to the tank. The mean feed recovered (RC) in the test runs was 0.84 ± 0.01 (n = 12).

After each meal, uneaten pellets were collected using a plastic strainer. Faeces were separated from waste feed items and discarded, and then excess water was removed. Finally, additional water was absorbed to paper, and the wet weight of the waste feed pellets calculated to the nearest gram.

2.3. Recovery from exhaustive exercise

In a continuation from the feed intake study, all fish were not fed for a period of 48 hours prior to being exposed to exhaustive swimming. The exhaustive exercise protocol has previously been described (Donaldson et al., 2010; Clark et al., 2012), and was designed to investigate whether triploid brown trout experience increased blood physiological disturbance (i.e. deviations from control diploids) when exposed to exercise associated with fisheries capture (e.g. catch-and-release) during a thermal challenge of either 10 °C or 19°C. We used a combination of water vortex and manual chasing for 10 minutes until all individuals were unable to continue burst type swimming. A water vortex was generated in each tank by opening the inflow to allow maximum inflow while allowing excess water to drain. This method was tested in three empty tanks prior to application within the trial to ensure a highly reproducible whirlpool in each tank. The uppermost temperature threshold was chosen on the basis of previously reviewed data (Ojolick et al., 1995; Hyndman et al., 2003; Atkins and Benfey, 2008). Oxygen saturation remained greater than 90 % (~ 9 mg / L) during the exercise protocol for 10 °C and 19 °C respectively.

2.4. Sampling procedure

Pre-exercise resting blood samples (Time '0') were taken on 11th February 2013 prior to exhaustive exercise with 5 fish / tank randomly sampled (n = 4 ploidy / temp). Thereafter, at 1; 4 and 24 hours after exhaustive exercise fish were rapidly caught and placed into a lethal

dose of MS-222 (100 mg/ L, Sigma-Aldrich, Poole, UK) then euthanased by cranial concussion. Thereafter, weight and length were measured for each fish before blood was withdrawn from the caudal vein using a 1 ml heparinised syringe (0.04 mg/L) and 23 G sterile hypodermic needle. Whole blood taken was placed into 1.5 mL microfuge tube and stored on ice before being centrifuged at 9676 x *g* for 3 min. The resulting plasma (~500 µL) was aliquoted into two micro-eppendorfs and snap-frozen using liquid nitrogen before storage at −70 °C for plasma analyses. This process was achieved in less than 5 minutes for all samples to eliminate changes, which may occur in blood chemistry post-sacrifice (Clark et al., 2011). Thereafter, blood sampling took place at 1, 4 and 24 hours post-exercise. All experimental work was carried out at the Institute of Marine Research Station, Matre, Matredal Western Norway. The work was conducted in accordance with the laws and regulations controlling experiments and procedures on live animals in Norway following the Norwegian Regulation on Animal Experimentation 1996 and reviewed by the University of Stirling Divisional Ethics Committee and Animal Welfare committee. No adverse effects were seen on the fish during experimental trials.

2.5. Blood haematology analysis

Plasma ions (Na^+, K^+ and Cl^-) were analysed by ion selective electrodes (ISE) using standard kits for the COBAS c111 auto analyser (Roche Diagnostics, Indianapolis, USA). Instrument accuracy was maintained by daily use of ISE maintenance and standard solutions (ISE Deproteinizer, ISE Etcher, Activator, and ISE Solution 1 and 2 to prevent the build-up of contaminants. For all other plasma parameters (lactate; glucose; pH; lactate dehydrogenase; phosphorus, calcium, magnesium; potassium; total protein, triglycerides; cholesterol; alkaline phosphatase; aspartate aminotransferase) the Maxmat (PL II) Multidisciplinary diagnostic biomedical analyser was used, while plasma pH was achieved by cell rupture, in a double

freeze-thaw protocol then pH read using a pH microelectrode (Thermo Scientific). Total plasma osmolality was measured using freeze point determination (Fiske micro-osmometer Model 210, Norwood, MA, USA) calibrated each day prior to use.

2.6. Radiography and vertebral deformities

After blood sampling diploid and triploid brown trout (n = 20 / tank) from each temperature and ploidy group (n =160 / ploidy) were individually labelled, stored on ice prior to being frozen on a flat surface at -20 °C for later radiography. Radiographs were taken using a portable X-ray apparatus (HI-Ray 100, Eickenmeyer Medizintechnik für Tierärzte e.K., Tuttlingen, Germany) and 30 × 40 cm sheet film (FUJIFILM IX 50, FUJIFILM Corp., Tokyo, Japan). The film was exposed at 12 mA s and 44 kV, and developed using a manual developer (Cofar Cemat C56D, Arcore (MI), Italy) with Kodak Professional manual fixer and developer (KODAK S.A., Paris, France). The pictures were digitized by scanning (Epson Expression 10000 XL, Seiko Epson Corp., Nagano-Ken, Japan). The vertebral column of each fish was examined using Adobe Photoshop CS2 and 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) (Appendix III). The number of vertebrae (V) per fish, the location and type of deformity were determined according to Witten et al. (2009).

2.7. Verification of ploidy

On 11 December 2012, prior to experimental set up, 25 fish per tank (n = 75 / ploidy) were randomly selected from each tank then euthanased prior to blood collected by caudal venipuncture. A drop of blood (~0.2 ml) was spread on a glass slide and allowed to air dry before being fixed by a 10 min immersion in 100 % methanol. The fixed blood smears were

then stained in 6 % Giemsa (Sigma) for 10 min (no cover-slip was added). The major axis diameter of 10 erythrocytes was measured from each blood smear taken using a light microscope fitted with a 1 mm graticule marked at 1 μm intervals (Image-Pro Plus, version 4.0, Media Cybernetics Silver Spring, MD, USA). All fish screened in each batch were shown to conform to the correct ploidy indicating 100 % triploid rate (Kruskal-Wallis test: $P < 0.05$).

2.7. Statistical analysis

Group mean and total feed intake per meal expressed as a percentage of estimated biomass were analysed using two-way GLM ANOVA (time*ploidy). Plasma parameters were analysed using a three-way mixed model GLM ANOVA (time * ploidy * temperature) over time. Post-hoc testing were achieved using Tukey's multiple comparison tests. Data was found to conform to normality and homogeneity of variance following Kolmogorov–Smirnov and Levene's tests and examination of residual plots. Feed intake data (% biomass) were arcine transformed prior to analysis and plasma parameter data, which did not conform, were subsequently log transformed prior to analyses. Fulton's condition factor (K) was calculated as $K = (100\ W) / FL^3$; where W is whole body-mass (g) and FL is fork-length (mm). Specific growth rate (SGR) over the experimental period was calculated as $SGR = 100\ (e^g - 1)$; where $g = (\ln W_f - \ln W_i) / (t_f - t_i)$, W_f and W_i are the mean final and initial W respectively and $(t_f - t_i)$ is the duration of the experimental period in days. The mean growth rate was also measured as the thermal growth coefficient (TGC) to account for any difference between ploidy groups as follows: $TGC = (W_f^{1/3} - W_i^{1/3}) \times (1000/DD)$; where W_f and W_i are as previously addressed for SGR and DD is the cumulative daily water temperature (°C). All statistical tests were performed using Minitab v16.1 with a significance level of $P < 0.05$. All results are presented as arithmetical mean ± SEM.

3. Results

3.1 Feed Intake and growth

Increasing the water temperature from ambient to 10 °C had a significant time and ploidy effect on feed intake for meals 1, 2 and 3 ($P < 0.05$; Fig 1A; B; and C). During the first meal, (Fig 1A) feed intake was greater than subsequent meals, except during day 12 when feed intake was reduced during meal 1 and increased in meal 2 (Fig 1B). Feed intake was highest during meal 1 and 2 and reduced during meal 3 (Fig 1C).

The thermal challenge for diploid and triploid brown trout to 19 °C occurred over a longer duration (2 °C / day: ~6 days). Increasing water temperature had a significant time and ploidy effect and an interaction of time and ploidy on feed intake for meal 1 and 3 (two way ANOVA, $P < 0.05$; Fig. 1D, E). The feed intake during meal 3 remained significantly higher in triploids than diploids at 19 °C ($P < 0.05$; Fig. 1F).

The total daily feed intake at 10 °C was not significantly different between ploidy at any time point (Fig. 1G). At 19 °C the total feed intake was significant higher in triploid than in diploids from day 16 - 24, however these differences were not significant at day 22 (Fig. 1H).

Prior to the start of the experiment triploid trout had a significantly heavier body mass, fork length and condition factor than diploids (Table 1A). At the end of the trial triploids remained heavier, longer in length and had lower condition factor than diploids. SGR did not differ irrespective of ploidy however TGC was higher in diploids at 10 °C but lower than triploid at 19 °C although not significant between ploidy (Table 1B).

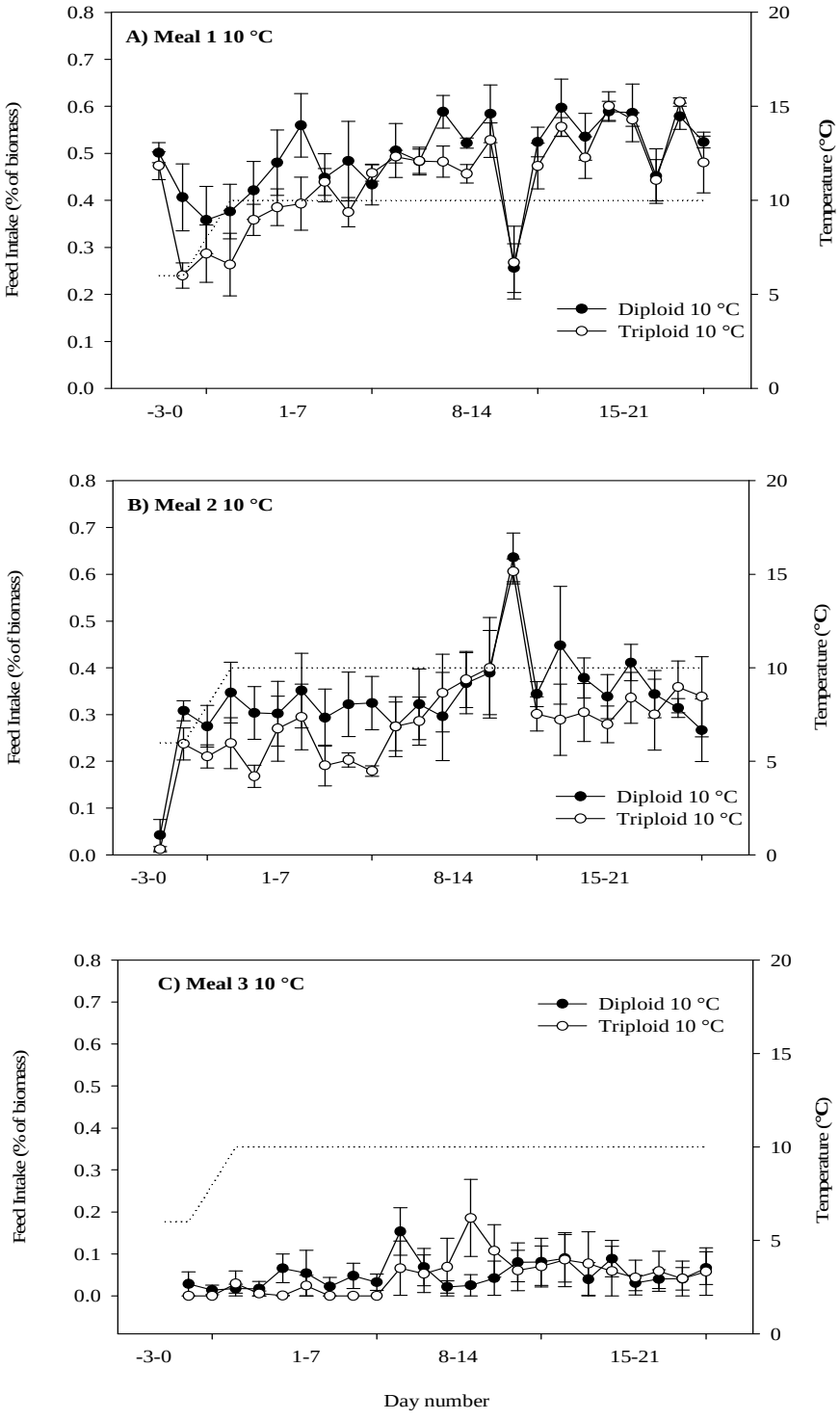

Figure 1a-c. Feed intake profiles of diploid and triploid brown trout (*Salmo trutta* L.) during the 24 day trial period. Data from the pre-experimental period (days -3 to 0) and the experimental period (day 1-21) are pooled and are presented as the mean ± SEM. All trout were reared in quadruplicate 455 L freshwater tanks at 6 °C then acclimated to 10 °C (2 °C/day) then fed to satiation three times daily during: A: 10 °C meal 1; B: 10 °C meal 2; C: 10 °C meal 3. Dashed line represents the temperature profile during the feeding study.

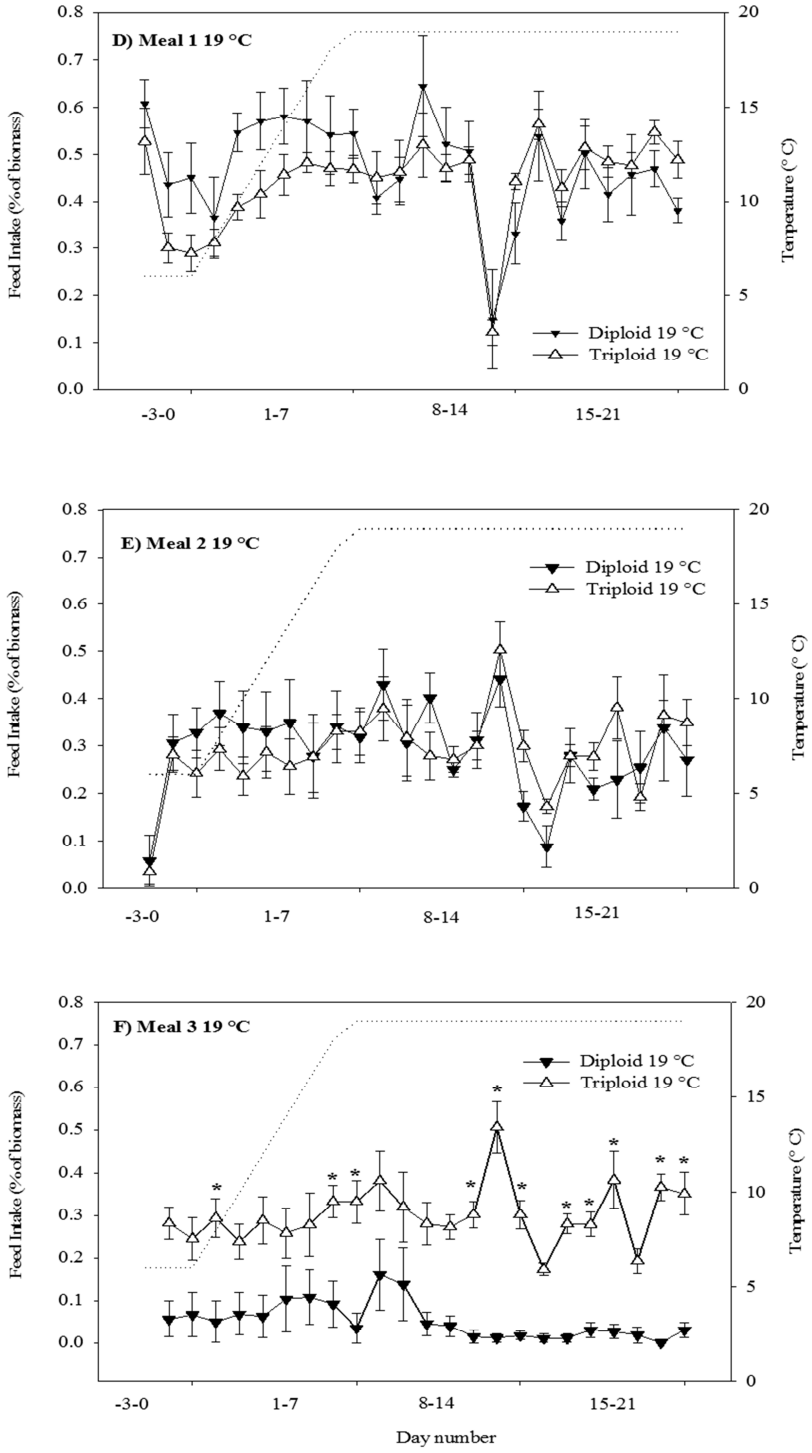

Figure 1d-f. Feed intake profiles of diploid and triploid brown trout (*Salmo trutta* L.) during the 24 day trial period. Data from the pre-experimental period (days -3 to 0) and the experimental period (day 1-21) are pooled and are presented as the mean ± SEM. All trout were reared in quadruplicate 455 L freshwater tanks at 6 °C then acclimated to 19 °C (2 °C/day) then fed to satiation three times daily during: D: 19 °C meal 1; E: 19 °C meal 2; F: 19 °C meal 3. Dashed line represents the temperature profile during the feeding study. Asterisk (*) indicate a significant difference between ploidy ($P < 0.05$).

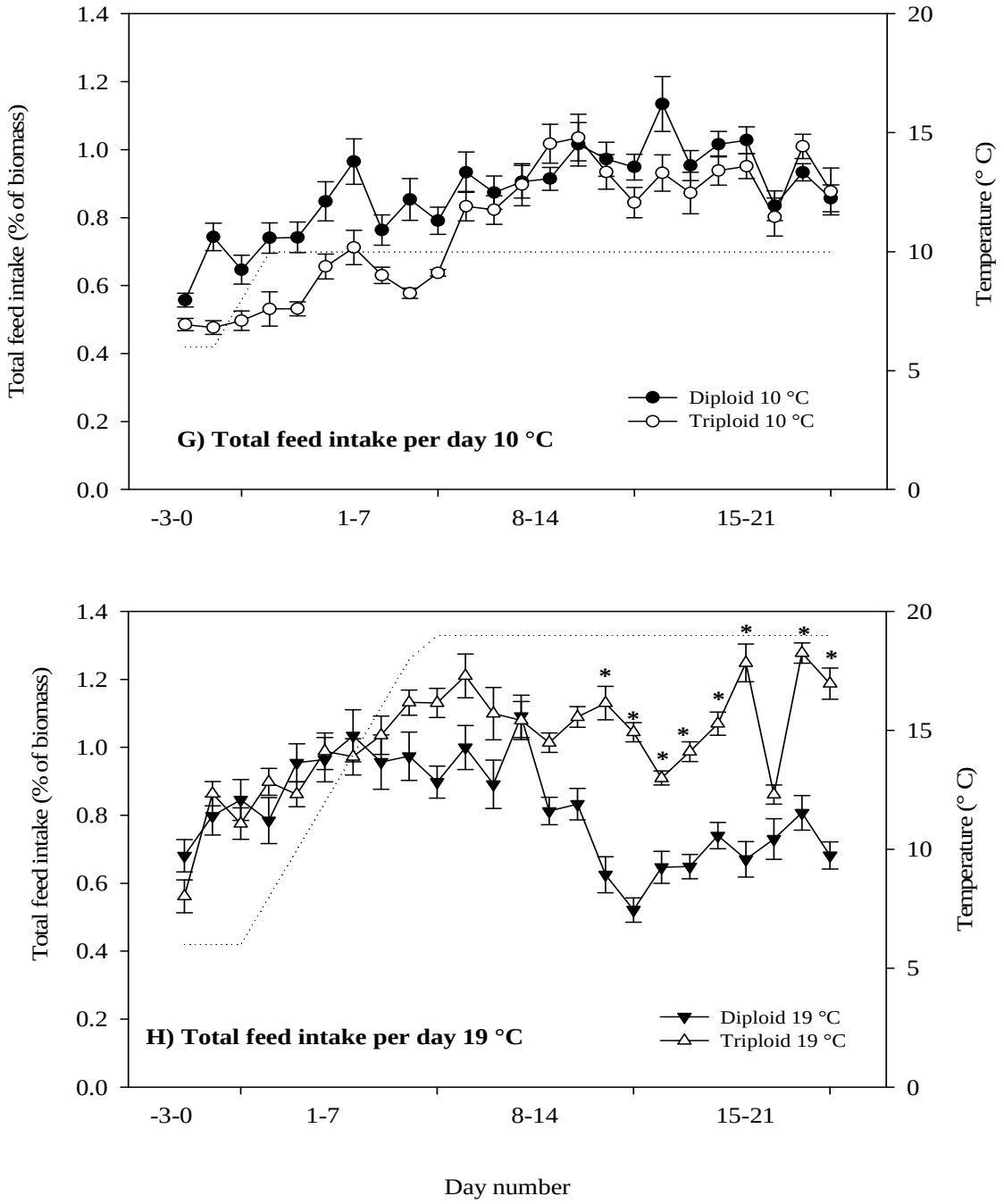

Figure 1g-h. Total feed intake (% of estimated biomass per day) profiles of diploid and triploid brown trout (*Salmo trutta* L.) during the 24 day trial period. Data from the pre-experimental period (days -3 to 0) and the experimental period (day 1-21) are pooled (n = 4) and are presented as the mean ± SEM. Total feed intake per day for 10 °C (G) and 19 °C (H) is the sum of meals 1-3 each day. Dashed line represents the temperature profile during the feeding study. Asterisk (*) indicates a significant difference between ploidy ($P < 0.05$).

Table 1. Growth performance (Mean ± SEM) of diploid and triploid brown trout at a) the start of the experiment, and b) after the swimming protocol (mean ± SEM). Prior to the start of the experiment 80 / ploidy / temperature were sampled for whole body weight (W); fork length (L); Fulton's condition factor (K). At sampling point b. specific growth rates (SGR) and thermal growth coefficients (TGC) were determined using (80 / temperature / ploidy). Different superscripts denote significant differences between treatment groups (ANOVA, $p < 0.05$).

Treatment		A. Start of experiment					
Temperature (°C)	Ploidy	N	W (g)	FL (mm)	K		
10	Diploid	80	70.1 ± 3.1^{a}	165.0 ± 2.2^{a}	1.54 ± 0.01^{a}		
10	Triploid	80	80.3 ± 3.5^{b}	174.1 ± 2.4^{b}	1.53 ± 0.01^{b}		
19	Diploid	80	70.1 ± 0.2^{a}	164.8 ± 2.0^{a}	1.54 ± 0.01^{a}		
19	Triploid	80	79.3 ± 3.5^{b}	173.5 ± 2.3^{b}	1.49 ± 0.01^{b}		
		B. Post-swimming protocol					
Temperature (°C)	Ploidy	N	W (g)	FL (mm)	K	SGR	TGC
10	Diploid	80	81.4 ± 3.6^{bc}	180.2 ± 2.5^{b}	1.36 ± 0.01^{a}	0.30 ± 0.03^{a}	0.46 ± 0.04^{a}
10	Triploid	80	91.3 ± 4.1^{a}	189.4 ± 2.5^{a}	1.31 ± 0.01^{b}	0.27 ± 0.02^{a}	0.40 ± 0.07^{a}
19	Diploid	80	76.0 ± 3.0^{c}	178.2 ± 2.1^{b}	1.32 ± 0.01^{b}	0.18 ± 0.06^{a}	0.15 ± 0.05^{b}
19	Triploid	80	87.1 ± 3.9^{ab}	187.0 ± 2.5^{a}	1.30 ± 0.01^{b}	0.21 ± 0.01^{a}	0.18 ± 0.01^{b}

3.2 Blood haematology

3.2.1 Lactate, glucose, pH, lactate dehydrogenase and total protein

Prior to the exercise protocol, basal lactate values obtained were not significantly different between ploidy at either 10 °C (diploid: 1.30 ± 0.11 mmol / L; triploid: 1.63 ± 0.13 mmol / L) or 19 °C (diploid: 1.98 ± 0.16 mmol / L; triploid: 2.15 ± 0.16 mmol / L) (Fig 2a). In both temperature groups, diploid and triploid plasma lactate increased significantly from basal levels post-exercise; however, values were not significantly different between ploidy for both temperature treatments ($P > 0.334$). The highest lactate values were observed in triploid 19 °C (6.52 ± 0.3 mmol / L) at 1 h post-exercise and the lowest in diploid 10 °C (4.38 ± 0.41 mmol / L). At 4 h post-exercise lactate levels decreased acutely and were not significantly different between ploidy or from basal levels although levels did remain significantly elevated from resting lactate levels in diploid 10 °C. All groups returned to near basal levels by 24 h post-exercise. A typical example of three-way ANOVA output corresponding to the lactate data is shown in Table 2.

Basal glucose levels were not significantly different between ploidy for both temperature treatments, but were generally lower at 19 °C than 10 °C, and increased post-exercise (Fig 2b). At 1 h after exercise plasma glucose were significantly elevated from basal levels in diploid and triploid 19 °C but not diploid and triploid 10 °C. At 4 h post-exercise glucose levels remained significantly elevated from basal levels in the triploid 10 °C (5.36 ± 0.20 mmol / L) and triploid 19 °C (5.21 ± 0.18 mmol / L) treatment groups. Thereafter, all treatments groups returned to near basal levels at 24 h post-exercise.

Prior to exercise plasma pH was lower in triploids for the 10 °C and 19 °C treatment groups, although these differences were not significant between ploidy (Fig 2c). At 1h post-exercise there was a marked decrease in plasma pH in all treatment groups, although differences were not significantly different from basal levels. Plasma pH values increased

significantly between 1 h and 4 h post-exercise in triploid 19 °C. At 24 h post-exercise plasma pH returned to near basal levels, with no significant differences between any treatment group.

Plasma LDH levels were elevated in both ploidy at rest in the 19 °C treatment group in comparison to the 10 °C treatment group, and there was an effect of the interaction between time and temperature (P = 0.001). However, differences observed were not significantly different between ploidy either pre-or post-exercise (P = 0.649; Fig 2d).

Plasma protein levels were not significantly different between ploidy or thermal regime at rest, and only triploid 19 °C showed a significant elevation above basal levels at 4 h post-exercise. At 4 h post-exercise triploid 19 °C had a higher protein level than diploid 19 °C although differences was not significant (Fig. 2e, P = 0.249). Plasma protein returned to near basal by 24 h post-exercise with no differences observed between ploidy or temperature groups.

Table 2

A typical example of a three-way GLM ANOVA performed on lactate data using the method of sequential sums of square for test. The fixed factors within the model were ploidy (2N or 3N) and temperature with replicate tank nested within temperature–ploidy grouping. DF: degrees of freedom, Seq.SS: sequential sums of squares, Adj MS: adjusted sequential mean squares, F: variance and P: probability.

Source (Lactate)	DF	Seq SS	Adj SS	Adj MS	F	P
Time	3	10.9	10.9	3.36	116.4	0.001
Ploidy	1	0.8	0.8	0.84	27.08	0.001
Temperature	1	0.4	0.5	0.47	15.2	0.001
Time*Ploidy	3	0.1	0.1	0.03	1.17	0.334
Time*Temperature	3	0.28	0.3	0.09	3.02	0.042
Ploidy*Temperature	1	0.01	0.0	0.01	0.32	0.573
Replicate (Ploidy*Temperature)	12	0.3	0.3	0.02	0.93	0.532
Time*Ploidy*Temperature	3	0.08	0.1	0.02	0.95	0.426
Error	36	1.1	1.1	0.03		
Total	63	14.1				

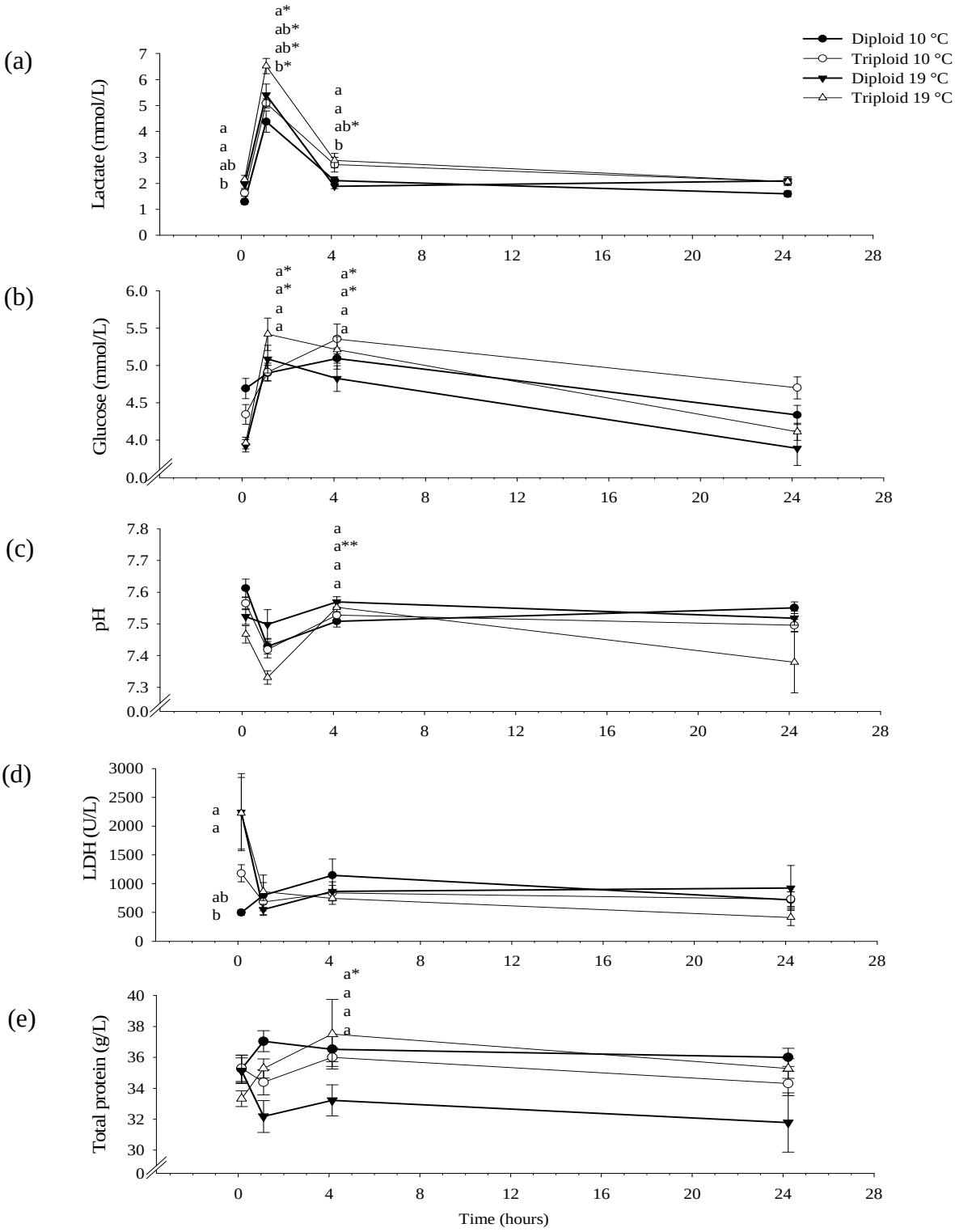

Figure 2. The effect of temperature, ploidy and exhaustive exercise on blood haematology of diploid and triploid brown trout at 10 and 19 °C for (a) lactate (b) glucose (c) pH and (d) lactate dehydrogenase and (e) total protein plasma levels. Pre-exercise (basal) samples are represented at time 0. After the exercise protocol blood samples were collected at time 1, 4 and 24 h post-exercise. At all-time points 20 fish/ploidy/temperature were sampled (mean ± SEM). Different letters indicate significant differences between ploidy and temperature at a given time-point. Asterisk (*) indicate a significant difference to basal levels and (**) indicate a significant difference between 1 and 4 h post-exercise within a given ploidy-temperature regime ($P < 0.05$).

3.2.2 Triglycerides, cholesterol, osmolality, Na^+ and Cl^-

Basal plasma triglycerides were not significantly different between ploidy or temperature regime. At 1 h post-exercise levels were significantly reduced below basal in diploid and triploid 10 °C (Fig. 3a, $P < 0.05$). By 24 hr post-exercise, diploid 10 °C remained significantly lower than basal levels.

Basal cholesterol levels were not significantly different between ploidy or thermal regime before exercise. 1 hr post-exercise triploid 19 °C (10.69 ± 0.41 mmol / L) had a significantly higher plasma cholesterol level than diploid 19 °C (8.36 ± 0.36 mmol / L; $P < 0.05$) (Fig. 3b). Plasma cholesterol levels did not differ between ploidy in the 10 °C treatment group at this point. 4 h post-exercise values were near basal, however, remained lower in the diploid 19 °C group with this trend continuing through to 24 h post-exercise where there an effect of ploidy ($P < 0.05$).

At rest, plasma osmolality values were not significantly different between ploidy within a given thermal regime, but were significantly lower in the 19 °C group (Fig 3c). Post-exercise, there was no significant difference between ploidy in the 10 °C treatment group pre- or post-exercise (diploid: 354.60 ± 3.81 mOsm / kg; triploid: 361.63 ± 6.38 mOsm / kg). By contrast, in the 19 °C treatment at 1 h post-exercise, triploids showed significantly elevated plasma osmolality values from resting levels, and was also significantly higher than diploid 19 °C (diploid: 337.10 ± 2.70 mOsm / kg; triploid: 371.72 ± 7.14 mOsm / kg, $P < 0.05$). At 4 h post-exercise plasma osmolality levels had returned to near basal levels in treatment 10 °C (diploid: 360.45 ± 10.58 mOsm / kg; triploid: 352.55 ± 8.24 mOsm / kg) and 19 °C (diploid: 331.74 ± 2.75 mOsm / kg; triploid: 324.20 ± 4.46 mOsm / kg) and were not significantly different between ploidy thereafter.

Plasma sodium levels did not differ between ploidy and temperature at any time point ($P = 0.727$, Fig. 3d).

Plasma Cl^- levels increased post-exercise, but did not differ significantly from basal levels in either treatment group and returned to near basal levels at 4 h post-exercise (Fig. 3e). Levels increased in all treatment groups at 24 h post-exercise albeit increases observed were not significantly different to basal levels.

3.2.3 P, Ca, Mn, K, ALP and ASAT

Temperature significantly affected plasma phosphorus levels, with basal plasma levels significantly lower at 19 °C than 10 °C treatment groups, although there was no difference between ploidy within a given thermal regime (Fig. 4a; $P < 0.05$). Post-exercise levels decreased in all treatment groups (1-4 h post-exercise) with differences being significantly lower from basal levels in triploid 10 °C at 4 h post-exercise, and diploid/triploid 10 °C at 24 h post-exercise. Similarly, in treatment 19 °C at 4 h post-exercise, plasma levels were significantly lower than basal levels in triploid however, differences were not significantly different between ploidy within temperature groups during the recovery period (1 - 24 h post-exercise; $P = 0.234$).

Plasma calcium levels were not significantly different between ploidy in either treatment at rest or post-exercise, however, levels did increase acutely in all treatment groups 1 h post-exercise (Fig. 4b). Plasma calcium values were significantly higher than basal levels for diploid and triploid at 19 °C (diploid: 119.83 ± 4.36 mg / L; triploid: 128.30 ± 3.37 mg / L; $P < 0.05$), while, differences were not significant at 10 °C in either ploidy. Thereafter, calcium levels returned to near basal levels in all groups with differences observed not significant between any ploidy groups (Fig. 4b, $P = 0.261$).

Basal plasma magnesium levels were not significantly different between ploidy groups either before or post-exercise, however, there appeared to be a significant effect of temperature with values consistently higher in triploid 19 °C (Fig. 4c, $P < 0.05$).

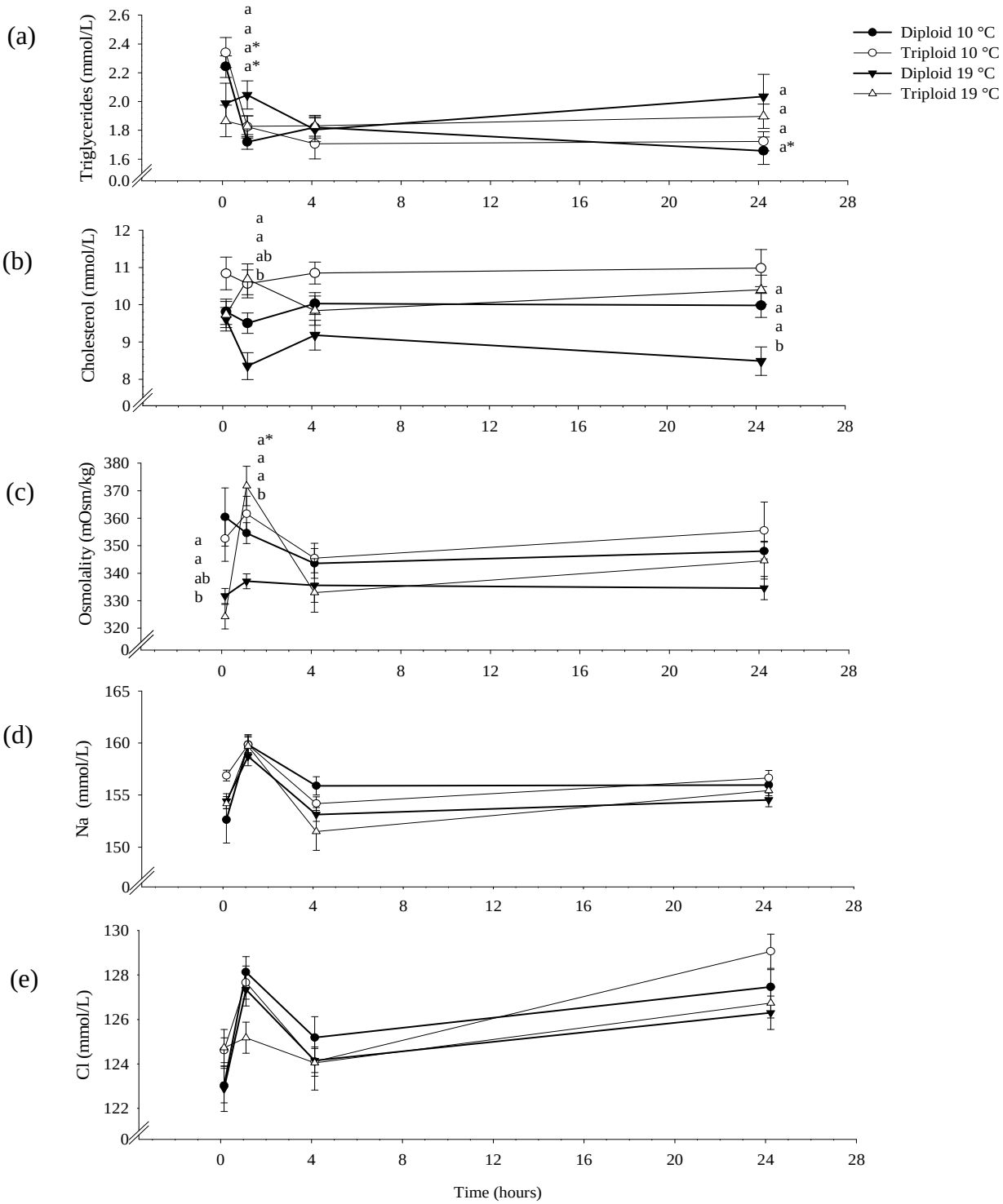

Figure 3. The effect of temperature, ploidy and exhaustive exercise on blood haematology of diploid and triploid brown trout at 10 and 19 °C for (a) triglycerides (b) cholesterol (c) osmolality (d) sodium and (e) chloride plasma levels. Pre-exercise (basal) samples are represented at time 0. After the exercise protocol blood samples were collected at time 1, 4 and 24 h post-exercise. At all-time points 20 fish/ploidy/temperature were sampled (mean ± SEM). Different letters indicate significant differences between ploidy at a given time-point. Asterisk (*) indicate a significant difference to basal levels within a given ploidy-temperature regime ($P < 0.05$).

Basal plasma potassium levels were generally higher in the 19 °C treatment (diploid: 4.27 ± 0.20 mmol / L; triploid: 4.83 ± 0.35 mmol / L) than 10 °C (diploid: 4.27 ± 0.20 mmol / L; triploid: 4.83 ± 0.35 mmol / L) although differences were not significant ($P = 0.297$). Post-exercise there was no significant differences between ploidy or within any treatment group (Fig. 4d, $P < 0.02$).

Basal alkaline phosphatase (ALP) levels were elevated at 10 ˚C (diploid: 277.39 ± 10.13 U / L; triploid: 298.43 ± 18.21 U / L) in comparison to 19 ˚C (diploid: 198.63 ± 12.29 U / L; triploid: 168.98 ± 12.19 U / L, $P < 0.05$, Fig. 4e) but did not differ between ploidy. Plasma ALP levels did not differ to basal levels at any point post-exercise at 10 ˚C in either ploidy. At 19 ˚C plasma ALP levels did not differ between ploidy post-exercise, although at 24 h post-exercise levels were significantly lower than basal in diploids.

Plasma aspartate aminotransferase (AST) levels differed significantly between temperature treatments but not ploidy both pre-and post-exercise (Fig. 4f, $P < 0.05$). Basal plasma levels were higher in treatment 19 °C (diploid: 651.59 ± 43.83 U / L; triploid: 694.23 ± 24.65 U / L) than 10 °C (diploid: 416.63 ± 33.83 U / L; triploid: 464.83 ± 29.00 U / L) but did not differ between ploidy within a given temperature regime at any time point (Fig. 6b). AST levels appeared highest in triploid 10 °C at 4 hr post-exercise (751.83 ± 25.02 U / L) and lowest in diploids at 10 °C (420.11 ± 17.81 U / L) 4hr post-exercise. Post-exercise AST levels did not differ appreciably from basal at any time point.

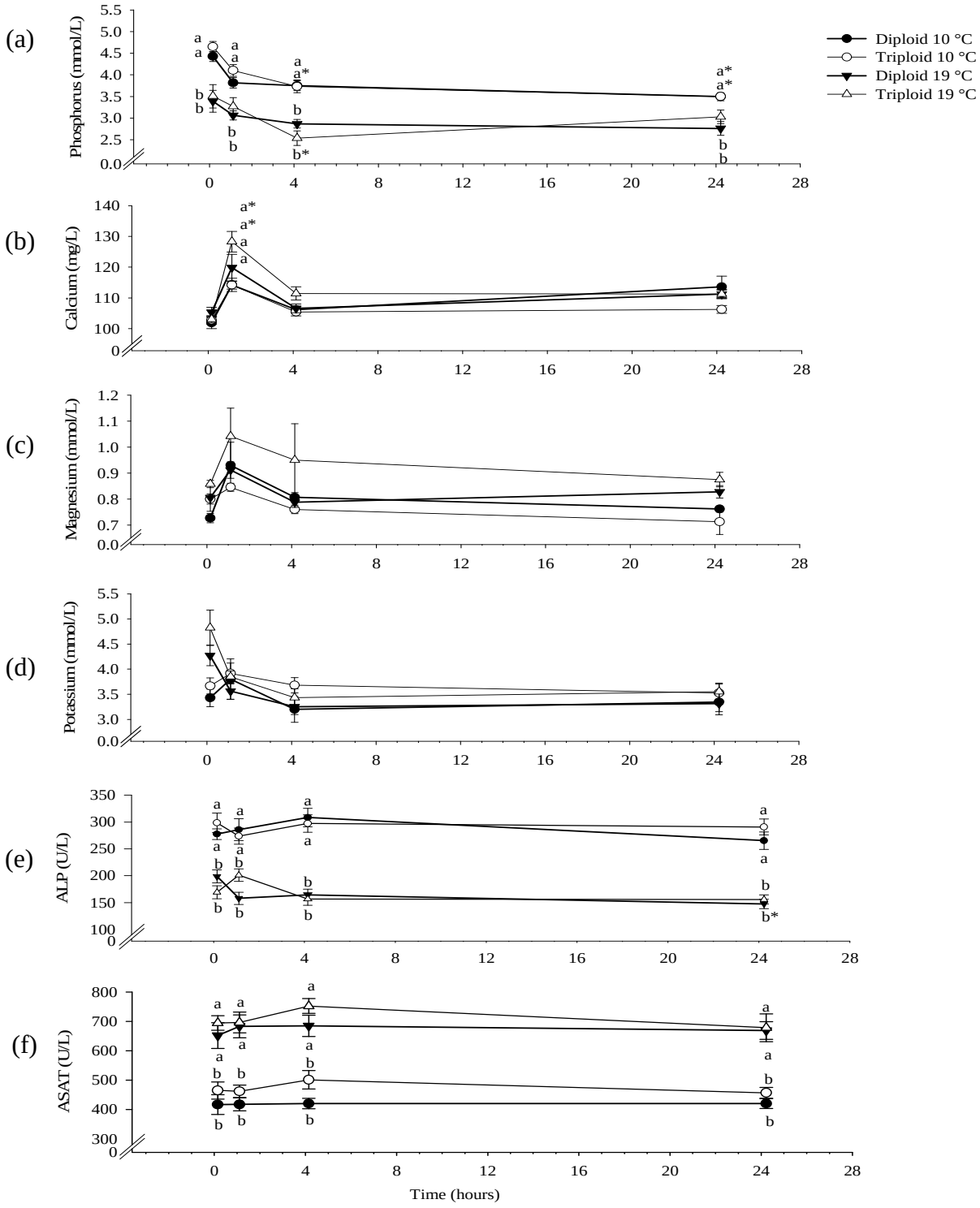

Figure 4. The effect of temperature, ploidy and exhaustive exercise on blood haematology of diploid and triploid brown trout at 10 and 19 °C for (a) phosphorus (b) calcium (c) magnesium and (d) potassium (e) alkaline phosphatase and (f) aspartate aminotransferase plasma levels. Pre exercise samples were taken at Time 0. After the exercise protocol blood samples were collected at time 1, 4 and 24 h post-exercise. At all-time points 20 fish/ploidy/temperature were sampled (mean ± SEM). Different letters indicate significant differences between ploidy at a given time-point. Asterisk (*) indicate a significant difference to basal levels within a given ploidy-temperature regime ($P < 0.05$)

3.3 Vertebral deformity

Vertebrae counts for diploid trout (58.1 ± 0.6) were not significantly different to their triploid siblings (58.0 ± 0.6, Kruskal-Wallis test H = 0.18; DF = 1; *P* = 0.673). Diploids had fewer fish (7 %) with radiologically deformed vertebrae (dv1+) than triploids (14 %) (Table 3; Appendix III indicates a typical diploid and triploid radiograph during this study). Both ploidies showed few individuals (< 1.9 %) with severe numbers of deformed vertebra (dv>10). In diploids, 65 of 9290 (0.7 %) vertebrae showed detectable deformities compared to 91 of 9286 (1.0 %) triploid vertebra. X-ray radiography revealed that in diploid trout the greatest prevalence of deformed vertebrae were in the tail area region (R3) and tail fin region (R4), whilst triploids also showed elevated deformity in cranial trunk (R1) and caudal trunk (R2) (Fig. 5). In diploids, vertebra 55 and 56 were the most affected vertebrae with a prevalence of 7.1%, while in triploids v2 was the most affected with a prevalence of 6.6 %. Out of 20 possible classifications of deformity type based on Witten et al. (2009), seven pathology types were observed in diploids and nine in triploids (Table 4). In diploids, 72 % of all observed deformities were associated with decreased intervertebral space, principally located in R3 and R4. In triploids, decreased intervertebral space; compression and decreased intervertebral space; compression without x structure; one-sided compression; compression and fusion and widely spaced and undersized accounted for 88% of all pathologies observed.

Table 3. Observed severity of deformed vertebrae in diploid and triploid brown trout characterised by radiology (n = 160 fish radiographed/ploidy). Values are expressed as the total number of individuals (%) within each ploidy grouped according to the total number of deformed vertebrae present (no. dV = number of deformity).

Severity	no. dV	Diploid	Triploid
No deformity	0	93.13	86.25
Mild	1-5	5.63	10.63
Moderate	6-10	1.25	1.25
Severe	>10	0	1.88

Table 4. Observed prevalence, type and localization of vertebra deformity in diploid and triploid brown trout characterised by radiology. Values are expressed as percentage of the total number of deformed vertebrae observed within each ploidy with n = 160 fish radiographed/ploidy. See Fig. 5 for a description of the different vertebral column regions (R). InterV: intervertebral. For each ploidy group, the two main types 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
Type of vertebral deformity (%)										
Decreased interV. space	3	12	**43**	14	72.3	0	4	**10**	0	14.3
Homogeneous compression	**5**	0	0	0	4.6	**2**	0	0	0	2.2
Compression and decreased interV. space	0	0	0	2	1.5	**11**	3	0	0	14.3
Compression without x structure	-	-	-	-	-	4	**8**	0	1	13.2
One-sided compression	0	0	0	**8**	7.7	2	**4**	0	**4**	11.0
Compression and fusion	0	0	0	**3**	3.1	3	**7**	0	4	14.3
Complete fusion	0	0	0	**9**	9.2	2	0	0	**7**	8.8
Widely spaced and undersized	-	-	-	-	-	9	**12**	0	0	20.9
Internal dorsal or ventral shift	0	0	0	**2**	1.5	0	0	0	**1**	1.1
Total (%)	8	12	43	37	100.0	34	38	10	18	100.0
Total (n)	5	8	28	24	65	31	35	9	16	91

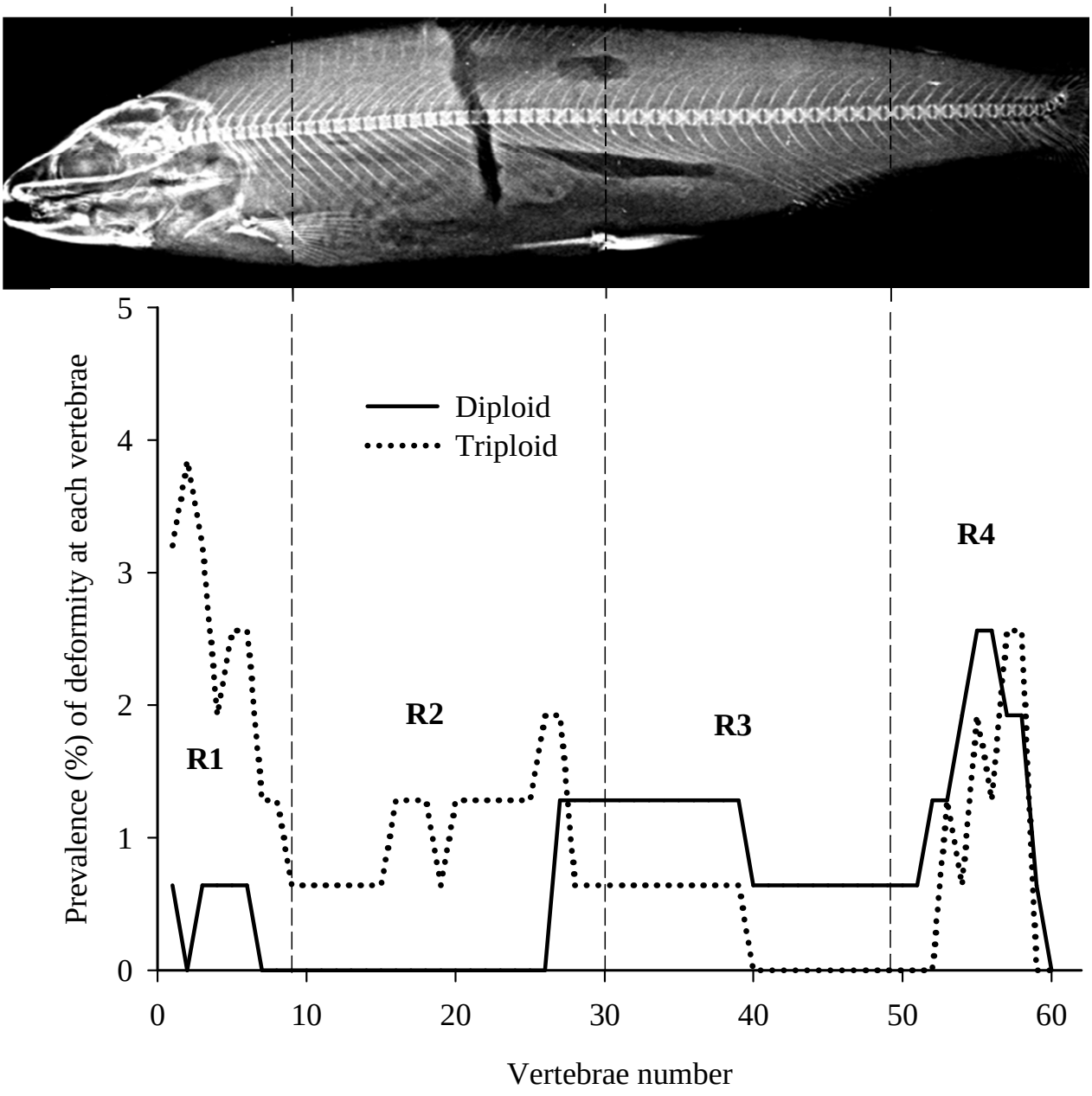

Figure 5. Prevalence (%) and localization of deformed vertebrae in diploid and triploid brown trout (based on the total number of deformed vertebrae within each ploidy; 65 and 91 deformed vertebra in diploid and triploid respectively from 160 radiographed / ploidy). The different regions of the vertebral column are shown as follows: R1 (cranial trunk, V1→ V8), R2 (caudal trunk, V9→ V30), R3 (tail, V31→ V49) and R4 (tail fin, V50→ V59) with R: region and V: vertebrae. Radiograph above indicates vertebrae positions of R1→ R4.

4. Discussion

4.1 Feed intake during increased acclimation temperature

At 10 °C feed intake profiles were not significantly different between ploidy during any meal fed which agrees with the study of Garner et al. (2008) where feeding and growth rates between diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*) were not significantly different. Similarly, Leclercq et al. (2011) indicated that there was no difference in growth between ploidy in Atlantic salmon (*Salmo salar* L.) post-smolts. In this study, differences in feed intake between ploidy were not expected at 10 °C as this is within the thermal optima for the species, as indicated by Elliott (1995). Previously, the temperature reported for maximal growth in brown trout was 16.9 °C; at a latitude of 43° N (Ojanguren et al., 2001). In addition, values reported for Norwegian stocks were higher (16.0 °C) at a latitude of 61° N (Forseth and Jonsson, 1994) than have been reported for British stocks e.g. 13.1 °C at a latitude of 54° N (Elliott et al., 1995). However, the results of feeding at high temperature (19 °C) were not expected as this is out of the range of brown trout thermal optima. In addition, triploid brown trout appeared to take longer to reach satiation than diploid brown trout indicating possible differences in digestion between ploidy. Previously, triploid rainbow trout have been shown to display greater feed intake and specific growth rates than diploids (Cleveland & Weber, 2013). The higher feed intake in triploids may relate to lower metabolic rates previously observed in triploid salmonids when reared at high temperature (Atkins and Benfey, 2008), suggesting that triploids may need to consume more food than diploids to meet physiological demands of the environment in which they inhabit. On the other hand, it has been shown that juvenile triploid Atlantic salmon contain increased total gut microbiota levels compared with diploid, with increases in *Pseudomonas spp*., *Pectobacterium carotovorum*, *Psychrobacter* spp., *Bacillus* spp., and *Vibrio* spp., respectively (Cantas et al., 2011). Gut microorganisms have the potential to influence weight gain and fat

deposition, and therefore subtle changes to gut microbiota composition influence energy expenditure, satiety and food intake (Flint, 2011). Differences in micro biota could influence the digestion and uptake of micronutrients between ploidy; however, this has not yet been fully elucidated.

It has previously been demonstrated that feeding rates of triploid salmon do not differ to feeding rates of diploids when reared in isolation (Taylor et al., 2013). Results of this study indicate that total feed intake was similar between ploidy and that at high temperature a gradual decline in total feed intake was observed. The negative effect of high temperature has been demonstrated in Atlantic salmon whereby a reduction in overall feeding and growth rate was observed when fish were kept at 18 °C for 3 months (Kullgren et al., 2013). Similarly, in this study the greatest decline of total feed intake at high temperature were observed in diploid trout and not in the triploids. These results suggests that triploid brown trout will feed equally or better than diploid brown trout at high temperature, and that other environmental factors (e.g. oxygen saturation) may be responsible for alterations in feeding and negative growth rates observed in previous studies on triploids (Ojolick et al., 1995; Altimiras et al., 2002). In a recent study, Preston et al. (2014) observed little difference in surface feeding response between diploid and triploid brown trout within an experimental stream although it was shown that diploid trout were more aggressive during diploid vs. triploid allopatric pairwise matchings. This may have implications after fishery stocking as triploid trout may be forced to inhabit less favorable environmental conditions in the presence of diploid brown trout when placed into sporting fisheries.

4.2 The effect of exhaustive exercise on blood haematology

There are very few published studies, which report the haematological changes between ploidy in response to exhaustive exercise (Hyndman et al., 2003a; Beyea et al., 2005). In this

study, we used a combination of tank vortex and manual chasing to elicit burst type swimming associated with a fisheries capture. Our results suggest that blood haematology can change drastically in diploid and triploid brown trout in response to exhaustive exercise.

Basal plasma lactate and glucose concentrations did not differ between ploidy at either temperature and values were comparable to those from other studies (Kieffer et al., 1994; Sadler et al., 2000; Hyndman et al., 2003a). In addition, at each temperature regime the typical secondary metabolic response to exhaustive exercise was exhibited, as indicated by elevated plasma glucose and lactate concentrations (Barton, 2002; Iwama et al., 2004; Portz et al., 2006; Li et al., 2011). The elevation of circulating plasma glucose suggests an increase in stress hormones such as catecholamines and cortisol, which speed the mobilization of glucose to support increased energy demands during increased exercise (Clarkson et al., 2005). Post-exercise the generated lactate in triploids appeared higher than diploids for a given temperature, and triploid trout appeared to clear lactate less quickly than diploid trout. Hyperlactatemia that occurs in triploids during sustained swimming could also be evidence of decreased blood lactate clearance. Blood lactate concentrations are the result of the balance between lactate clearance and production. In rainbow trout (*Oncorhynchus* mykiss) 10 – 20 % of the total lactate produced after exercise is released from the blood to the muscle. In addition a large proportion of the lactate released into the blood is used as an oxidative fuel for other tissues such as the heart and the red muscle (Milligan and Girard, 1993). However, previous studies suggest that lactate clearance was quicker in triploid than diploid brook trout (Hyndman et al., 2003a). In other species, Beyea et al. (2005) reported that triploid Short-nose sturgeon (*Acipenser brevirostrum*) had higher plasma lactate values following acute handling stress. In the present study, the more efficient clearance of lactate in diploid trout may reflect an enhanced aerobic capacity, as total surface area available for gaseous exchange is reduced

in triploids, and the maximum internal erythrocytic diffusion distance likely increased (Benfey, 1999).

Of note in the present study, it appeared that plasma pH was closely related to the severity of the short-term exercise in brown trout. Basal plasma pH did not differ between ploidy, although triploids generally had lower plasma pH values for a given temperature. However, after ten minutes of exhaustive exercise, triploids appeared to have a lower plasma pH than diploids indicating greater plasma acidosis for a given temperature. In previous studies blood pH following fishing capture was 7.2 in blue marlin *Makaira nigricans* (Dobson et al., 1986); 7.5 in tuna *Katsuwonus pelamis* (Perry et al., 1985); 7.4 in rainbow trout *Oncorhynchus mykiss* (Turner et al., 1983) and 7.4 in snapper *Pagrus auratus* (Wells and Dunphy, 2009). In the present study the lowest plasma pH observed after exhaustive exercise was in triploids at high temperature. In other species such as tuna (*Thunnus albacares*), which require high rates of O_2 delivery to tissues after exhaustive exercise, plasma pH may be reduced by as much as 0.4 pH units (Lowe et al., 1998). In many teleosts, a reduction in blood pH reduces both hemoglobin-oxygen (Hb-O_2) affinity and O_2 carrying capacity due to the Root effect (Root, 1931), and has a role in delivering O_2 to the retina and swimbladder (Scholander & Van Dam, 1954; Wittenberg & Wittenberg, 1974; Rummer et al., 2013). It has been identified that these tissues possess specialized acid-producing cells in conjunction with a dense counter-current capillary network (rete) that localize and magnify acidosis, thus promoting O_2-offloading via the Root effect (Scholander & Van Dam, 1954; Wittenberg & Wittenberg, 1974; Rummer et al., 2013). Bernier et al. (2004) found that triploid Chinook salmon had reduced arterial O_2 content in comparison to diploids during sustained swimming trials. This concurs with previous work by Graham et al. (1985) who found a reduced Hb-O_2 loading ratio in Atlantic salmon blood. Although the O_2 carrying capacity of diploid or triploid brown trout was not determined in this study, lower plasma pH of triploids during exhaustive exercise may reduce

the affinity of blood for O_2 (Bohr effect) therefore impacting on the maximal carrying capacity (Root effect). During such circumstances, the resulting acidosis contributes to the fixed acid Bohr Effect, with the principal consequence being a reduction in the arterio-venous pH gradient and persistently low arterial pH (Wells and Dunphy, 2009). While in the current study differences in plasma pH were modest between ploidy, in other studies small deviations in pH can have large effects on oxygen carrying capacity of the blood in salmonids (Pelster & Weber, 1990; Brauner & Randall, 1996; Bernier et al. 2004).

Exhaustive exercise can also disrupt ionic and osmotic balances within the blood and tissues by affecting water or ion uptake or other transport mechanisms (Wendelaar Bonga, 1997). In this study we observed an increase in plasma sodium and chloride ions post-exercise, which suggests that an accumulation of metabolites in the muscle resulted in an osmoregulatory disturbance in the plasma. As a result, water may have moved from the plasma into the surrounding muscle tissue causing an increase in the plasma ion concentration (Wood, 1991). Previous studies have shown that sockeye salmon (*Oncorhynchus nerka*) had increased plasma ion concentrations of sodium and chloride 30 minutes after a simulated capture event (Gale et al., 2011). In the present study, our values suggest that diploid and triploid brown trout underwent some osmoregulatory disturbance during exhaustive swimming, although based on previous data these values seem within the normal range for salmonids (Clark et al. 2012). However, it would appear, at high temperature, triploids had significantly higher osmolality values than diploids after exercise indicating greater osmoregulatory disturbance at this temperature.

Diploid and triploid brown trout showed similar basal plasma cholesterol and triglyceride concentrations. In Arctic charr (*Salvelinus alpinus*), similar differences in plasma cholesterol concentrations were found between GH transgenics and control, with transgenic fish having reduced plasma triglyceride concentrations (Krasnov et al., 1999). In the present study, it

appears that diploid and triploid brown trout plasma cholesterol and triglyceride levels were non-responsive to exhaustive exercise. However, an interesting result of the present study was that plasma cholesterol concentrations in diploid trout decreased 1 hr post-exercise and then returned to basal levels after 4 hr. Circulating lipids originate from three sources: newly absorbed from food, recently processed in the liver and then transported to the blood or mobilized from storage sites (Sheridan, 1988; 1990). In this study, experimental fish were starved for 48 hr prior to exhaustive exercise; therefore, the experimental fish would have to rely on oxidation of lipid released from muscle and adipose tissue to fuel the recovery process (Wang et al., 1994; Keins and Richter, 1998; Richards et al., 2002a, b; Li et al., 2011). Therefore, in light of no clear differences between plasma triglyceride and cholesterol concentrations in diploid and triploid brown trout this may indicate that these fish mobilize similar lipids to meet the increase in energy demand from the exhaustive exercise and that similar esterification of nonesterified fatty acids into triglyceride occurred in the blood (Li et al., 2011). Taken together these findings may also suggest that diploid and triploid brown trout show little reliance on lipid (especially triglyceride) to fuel basal and post-exercise recovery processes. However, further work on muscle metabolism to determine the precise roles of lipids and reduced surface to volume ratio on muscle recovery in triploid brown trout is required.

There was little difference in deformity prevalence between diploid and triploid brown trout in the present study. This is in contrast to many studies in salmonids that have reported increased prevalence of morphological deformities in triploids leading to lower survival rates and growth during early development (Benfey, 1999; Sadler et al., 2001; Cotter et al., 2002; Preston et al. 2013; Fraser et al., 2013). In this study diploid and triploid trout displayed a low detectable prevalence of vertebrae deformities (diploid: 0.7 %, triploid: 1.0 %). Interestingly, the type and the location of deformity observed differed between ploidy, with 73.3 % of

deformity associated with decreased intervertebral space in diploids principally located within the tail region. On the other hand triploid deformity was characterised by a wider range of deformity type with decreased intervertebral space; compression and decreased intervertebral space; compression without x structure; one-sided compression; compression and fusion and widely spaced and undersized accounted for 88% of all pathologies observed. Deformity was principally located within the cranial trunk and caudal trunk areas in triploid trout as opposed to the tail region observed in diploids (Fjelldal and Hansen, 2010). The prevalence of deformities reduces the aesthetic and commercial value of the fish but could also affect swimming ability and subsequently the fighting qualities of a sport fish (Ornsrud et al., 2004, Powell et al., 2009). Overall, most studies showed that the causal mechanisms for deformity prevalence in triploids are physical, chemical or dietary-related. For example, cataracts were observed in all salmon transferred to sea however, scores were significantly lower in salmon fed histidine-enriched diets (Breck et al., 2005; Taylor et al., 2014b) indicating that triploid-specific diets have not yet been formulated for optimising growth and welfare in Atlantic salmon. The deformity types observed in triploid Atlantic salmon generally appear in saltwater and may also be an environmental effect of the marine growing conditions when these fish enter periods of fast growth (Leclercq et al., 2011). However, from this study it appears that deformity prevalence in diploid and triploid brown trout may be less of a concern compared to Atlantic salmon.

The results of this study indicate that diploid and triploid brown trout demonstrated similar feed intake profiles at lower temperature, however at high temperatures triploid trout feed intake was significantly higher than diploids. In addition, similar primary physiological response to increased acclimation temperature was observed between ploidy however, some secondary responses to exhaustive exercise of triploids were higher than diploids. This suggests that triploids may take longer to recover from exercise than diploids at higher

temperature after fisheries capture. This could have implications for the freshwater sport fisheries sector, which endorse catch and release fishing practices more specifically at high temperatures.

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CHAPTER 6: SUMMARY OF FINDINGS

In this chapter the main findings of each research chapter are summarized:

Chapter 2: ***OPTIMISATION OF TRIPLOIDY INDUCTION IN BROWN TROUT (Salmo trutta L.).***

- There is a wide window where triploid induction can be induced in brown trout from 275 – 425 centigrade temperature minutes post-fertilisation.
- High triploid rates (93. 6 – 100.0 %) can be induced through a range of pressure (9, 500 – 11 500 psi) and durations (4 - 7 mins).
- Higher survival and significantly lower deformity prevalence was observed in the optimised HP shock (10,000 psi (689 Bar) for 50 CTM duration at 300 CTMs post fertilisation) in comparison to the EA protocol.

Chapter 3: ***ASSESSING PLOIDY STATUS OF BROWN TROUT (Salmo trutta) USING IMAGE ANALYSIS, FLOW CYTOMETRY AND MICROSATELLITE MARKERS.***

- Highly polymorphic centromere-distant microsatellites identified ploidy levels with 100 % accuracy.
- Red blood cell measurements and flow cytometry are invasive, labour-intensive, or have costly equipment and maintenance and therefore not convenient for screening large numbers of commercial trout.
- Microsatellites provide an accurate, economical and non-invasive verification technique over more traditional techniques for assessing ploidy levels in brown trout.

Chapter 4: *SURFACE FEEDING AND AGGRESSIVE BEHAVIOUR OF DIPLOID AND TRIPLOID BROWN TROUT SALMO TRUTTA DURING ALLOPATRIC PAIRWISE MATCHINGS*

- Dominance hierarchies were observed in all diploid and triploid allopatric pairwise matches.
- Diploid trout were more aggressive than triploid trout in cross-ploidy matchings.
- Surface feeding increased when fish were conditioned to a floating diet.
- No difference in surface feeding behaviour was observed between diploid and triploid trout irrespective of diet fed.
- Sneak-feeding behaviour was observed in subordinate triploid trout in the presence of dominant diploid trout.

Chapter 5: *THE EFFECT OF TEMPERATURE ON FEEDING BEHAVIOUR AND BLOOD HOMEOSTATIS FOLLOWING EXHAUSTIVE EXERCISE IN DIPLOID AND TRIPLOID BROWN TROUT (Salmo trutta L.).*

- Triploid trout had significantly higher feed intake than diploids at higher temperature.
- Post-exercise triploids had higher levels of plasma lactate, glucose, lower plasma pH and significantly higher osmolality and cholesterol values than diploids.
- The faster clearance of lactate in diploid may reflect an enhanced aerobic capacity.
- Deformity prevalence was not significantly different between ploidy.

CHAPTER 7: GENERAL DISCUSSION

The overall objective of this thesis was to optimise the production and verification of triploidy and expand knowledge of brown trout behaviour and physiology. A methodological approach was taken to 1) improve triploidy production by optimising the hydrostatic pressure (HP) shock, and confirm ploidy status by an evaluation of the methods used to audit triploids, 2) assess behavioural differences that could impact on growth and stock-out performances when placed into freshwater fisheries with diploids, and 3) evaluate the environmental sensitivity of triploids, fundamental to improving the understanding of triploid performance in culture and within a freshwater fishery environment. The following general discussion will evaluate the main findings and the wider implications from the research work, and elaborate on the limitations of the experimental work undertaken, and identify where current research opportunities exist within these areas of scientific interest.

1. Induction and verification of triploidy in brown trout.

Research was undertaken to develop a robust triploidy induction protocol delivering 100 % triploid rate and high yield. In order for the induction protocol to be successful the pressure intensity and duration must be sufficient to prevent second polar body extrusion while allowing chromosomal division. In our studies, hydrostatic pressure (HP) shock was employed as opposed to temperature shock for several reasons. Firstly, HP shock is applied consistently throughout the pressure chamber giving uniform delivery as opposed to thermal shock which can lead to localised heating due to the insulating properties of the surrounding eggs (Malison et al., 2001). Secondly, HP shock is easier to deliver with a fine control over the ramping up and down of the pressure in the chamber and therefore less invasive than thermal shock, although there is the potential for more physical and mechanical handling when loading and unloading the pressure chamber. Recent studies comparing these two

methods indicated that survival rate was higher and morphological deformities lower using HP shock than thermal shock (Haffray et al., 2007). For these reasons, the experimental work was conducted using HP shock and focussed on the three main parameters known to influence the effectiveness of the shock on triploid yields e.g. timing, intensity and duration. Results of the timing experiment demonstrated that there is a wide effective window (275 – 425 CTM) where high triploid rates and yields could be achieved by using a 10,000 psi HP shock of 5 minutes duration. In contrast, the Environment Agency's recommended protocol resulted in high triploid rate (95.4 %) however the second lowest triploid yield of the shocks tested (82.4 %). Previously, Gillet et al. (2001) indicated that high triploid yields of 82 to 100 % could be obtained in Arctic charr (*Salvelinus alpinus*) from 240 – 400 CTM suggesting a wide window for triploidy induction. In our study the optimum time of pressure application was defined as 300 CTM which reliably delivered 100 % triploid rate and high yields (72 %). The wider window observed could be related to the slower rate of embryonic development in cold water species such as brown trout. In non-salmonid temperate species such as European catfish (*Silurus glanis*), the window of application of the shock appears much narrower probably due to warmer water and faster embryonic development (Linhart et al., 2001).

Since intensity is the second most important variable of pressure shock to induce triploidy after timing, the efficacy of the HP shock was further refined by fine-tuning HP intensity and duration. HP shocks applied for too long or at a too high intensity can both result in reduced survival in rainbow trout (*Oncorhynchus mykiss*) (Chourrout, 1984). Results from the second experiment indicated that high triploid yields are obtained when brown trout eggs are subjected to combinations of HP intensity from 9,500 - 11,000 psi and duration of the shock of 4 - 7 mins applied at 300 CTM. These results indicate the plasticity of brown trout eggs to both pressure duration and intensity as shown for timing. In other species such as Coho salmon (*Oncorhynchus kisutch*) it has also been demonstrated that HP shock is effective

at inducing triploidy, however survival was inversely correlated with the intensity of the treatment, and therefore triploid yields were lower (24.6 vs. 72.0 %) at higher pressure intensities (Teskeredzic et al., 1993). In addition, this study indicated that deformity prevalence was higher in treatments with greater pressures and/or longer durations. The highest deformity rate was observed in the EA protocol for both the timing and pressure and duration studies, significantly so in the former which indicates the adverse effects of combinations of high pressure intensities and excessive durations as previously observed in other salmonids (Teskeredzic et al., 1993).

Other factors than the HP shock parameters can greatly influence the triploidy induction process such as egg quality. When eggs are released from the follicles during ovulation, their ability to be fertilised and develop into a viable embryo decreases with time (Aegerter & Jalabert, 2004; Bobe & Labbe, 2010). This is known as post-ovulatory ageing or the over-ripening process. In fish, post-ovulatory ageing induces a loss of egg viability and embryonic survival (Kjorsvik et al., 1990) along with an increased occurrence of embryonic malformations and ploidy anomalies (Aegerter & Jalabert, 2004). Until recently, many triploid trout producers induced triploidy on egg batches of lesser quality collected at the end of the stripping season rather than during the peak season due to production imperatives dictating commercial targets are met first prior to trying something new, subsequently affecting triploid yields. Poor egg quality in triploid Atlantic salmon results in rapid egg drop out during embryogenesis (Taylor et al., 2011). In addition, other environmental factors (e.g. temperature) have also been shown to impact on fish embryo survival (Aegerter & Jalabert, 2004).

Interestingly, our results indicate that temperature and embryonic development are intrinsically linked and that by adjusting timing of application and duration relative to pre-shock temperature it was possible to effectively deliver the same optimised shock at a range

of temperatures. In addition, this indicates that shock duration in inversely proportional to temperature within the current range tested (6 – 12 ^{o}C) and pressure duration decreases as temperature increases. In hatchery-scale triploid production, maintenance of a specific pre-shock incubation temperature requires accurate and reliable water heaters with minimum change. Maintenance of the pre-shock incubation temperature recommended by the EA (10 – 11 ^{o}C) could require regular monitoring and adjustment of heating equipment to maintain stable regime, if incoming water supply is below or above this temperature. On the other hand, given that brown trout typically spawn at low temperature from 5 to 8 °C (Rustadbakken et al., 2004; Arslan & Aras, 2007; Piecuch et al., 2007) this would suggest that the use of lower temperatures during the induction process may be more applicable commercially. In addition, depending on latitude and seasonality, air temperature may cause heat-sink effects on the pressure chamber and incubator baths, resulting in unstable thermal profiles leading to suboptimal shock conditions. A further benefit of using ambient rather than artificially heated water baths for hatchery management is that post-HP-shock eggs will not be subjected to a further thermal stress if they are returned to hatchery water at <10 °C. Thermal shock of newly-fertilised eggs is known to increase rate of egg drop out, and increase the potential for deformity (Aegerter & Jalabert, 2004) subsequently impacting on productivity. In particular it has been demonstrated that sub-optimal egg incubation temperature affects deformity rate in triploid Atlantic salmon parr whereby deformity prevalence (~2, 6 & 16 %) increased linearly with temperature (6, 8 & 10 °C respectively) (Fraser et al., 2015). This evidence alone further advocates the use of ambient water supply and adjusting timing and shock duration, rather than artificially elevating water temperature for shock purposes to minimise thermal trauma to the developing embryos.

On a final note, the results of the current study indicated that triploid trout producers should adopt the refined protocol (10,000 psi, 5 mins duration, 300 CTM) developed in the

present study which appear to result in higher yields and lower deformity prevalence refining the triploidy induction process. These results indicate that the EA protocol appears to be beyond what is considered necessary for optimised triploid induction in brown trout, consequently leading to increased deformity rate and lowest survival rates at yolk sac absorption; however these differences were small. Similarly, the results of the triploid producers' survey carried out in 2010–2011 (Appendix I) indicated that farmers experienced increased deformity of commercial batches of triploids suggesting the adverse effect of the increased pressure intensity and duration when using the EA protocol however this may also be related to temperature management, genetics of the stock used and HP shock itself. That said it should be considered that climate change might impact by sufficiently raising ambient water temperatures to impact on ovulatory rhythms or spawning time and therefore the success of triploidy induction.

The induction of triploidy either experimentally or commercially must be followed by an accurate assessment of ploidy in the HP-shocked population since the induction process may not consistently deliver 100 % accuracy in all the individuals tested due to operator/mechanical failure. On the other hand, sometimes the induction of triploidy results in mosaics (Arai, 2001) in which ploidy level varies across tissues. In such cases, it is important to verify that germ cell precursors are triploid using flow cytometry or microsatellite markers to ensure sterility (Piferrer et al., 2009). For this reason it is envisaged that farmed brown trout intended for stocking purposes will be legally required to verify triploidy status prior to deployment within freshwater fisheries. Currently, there are no on-farm ploidy verification techniques employed by commercial brown trout producers and therefore a clear need for reliable verification techniques to support the sector and ensure compliance with legislation. In the second experimental chapter, 22 microsatellite markers were selected from the literature and assessed for accuracy at determining the ploidy status of brown trout (Appendix

II). The microsatellite markers were compared with other techniques, which are routinely used, namely red blood cell measurements and flow cytometry. Results of this study indicate that triploid verification by cellular and nuclear measurements is inexpensive and easy to use however, this method is labour-intensive and not suitable to early verification due to the requirements of blood or the mass screening of commercial brown trout due to the time taken to process samples. On the other hand, further development of this technique by utilisation of semi–automated software to measure red blood cell axes would greatly improve the speed of this technique. While this technique is accurate at resolving ploidy levels in small numbers of individuals using nuclear and cellular surface areas and volumes, this study demonstrated overlap in size measurements in nuclear and cell minor axes between ploidy suggesting reliability issues when minor axes are compared. On the other hand, flow cytometry was 100 % accurate at identifying ploidy status, indicating that triploid trout had 1.5 times greater DNA content than diploid trout. Flow cytometry appears accurate for assessing triploidy in brown trout and may be used on eyed-eggs as previously demonstrated (Leucommandeur et al., 1994). However, due to the high cost associated with equipment purchase and maintenance thereof, microsatellite markers would appear more applicable to the brown trout aquaculture and fisheries industry since they are cost-effective, reliable, and accurate and can verify ploidy at any stage of development. In addition, these microsatellite markers would allow long-term performance monitoring of commercial triploids by genotyping of fin clips at low cost and without sacrifice (non-invasive).

At present, there is no on-farm assessment of triploidy in brown trout. This negates early assessment of triploid status and therefore producers have to hold egg batches that may be suboptimal resulting in increased production costs. Also, many hatcheries have limited incubation facilities and later assessment may result in facilities being used for sub-optimal batches. If the induction process is not 100 % effective, commercial batches of putative

triploids will contain diploids which failed to respond to the pressure treatment. The microsatellite markers reported here would alleviate this bottleneck and allow verification of ploidy at an embryonic stage of development (i.e. eyed-ova stage), which is earlier than most other applicable techniques (except karyotype analysis, Crozier & Moffatt, 1989b). This would benefit triploid producers and allow large commercial batches of HP-shocked ova to be screened early, ensuring triploidy status prior to on-growing and sale. However, ensuring triploidy requires screening based on sample size determination. In order to determine the number of samples to screen from each batch (diploid prevalence), sample size should be determined according to the desired precision calculated based on an appropriate confidence interval. On the other hand, screening many hundreds of HP-shocked individuals within a batch to demonstrate a low diploid prevalence (<5%) may provide a diminished return for the effort involved, and may not be justified commercially.

The potential benefits of microsatellite markers as a cost-effective, reliable, and accurate method for identifying triploids is evident however the current study assessed ploidy in one strain of commercially-available all-female diploid and triploid brown trout. Further work is needed to identify the suitability of these markers to other genetic stocks of diploid and triploid brown trout, and whether these markers may be utilised to determine ploidy levels in other salmonids (e.g. rainbow trout and Atlantic salmon), in which triploidy is becoming commercially significant.

2. Surface feeding and aggressive behaviour in diploid and triploid brown trout

The behaviour of diploid salmonids is well documented; however, there are few studies, which have studied the behaviour of their triploid siblings and/or the interaction between ploidy. The research conducted (Chapter 4) was underpinned by earlier work by Carter et al. (1994) and McGeachy et al. (1995) who observed more severe fin damage in triploid Atlantic

salmon parr in the presence of diploids. Interestingly, the present study indicated that during a pairwise matching of all diploid trout, differential resource acquisition was evident with one dominant fish gaining all access to food items. In contrast, the interaction between ploidy indicated similar but asymmetric dominance hierarchies' form, with diploid trout more likely to dominate. However, the dominance of diploid over triploid fish did not translate into differential resource acquisition as shown in the all-diploid pairings. These observations suggest that during cross-ploidy interactions dominance does not confer preferential access to food and positional status within the stream. The importance of this work should not be understated as it provides the first indication of the potential behaviour of triploid brown trout once released into freshwater fisheries. Although this study was conducted within an experimental stream, the study describes the only clear visual insight into the interactions between ploidy. Previous studies by Deverill et al. (1999) and Hedenskog et al. (2002) indicated that hatchery brown trout were more aggressive than wild trout or that agonistic behaviour was higher among wild than sea ranched strains of brown trout respectively. More laboratory (flume) based studies are needed to determine the interactions between larger "stocked" triploid brown trout and small wild diploid conspecifics. These studies should determine whether the existence of an ecological advantage of wild diploid brown trout remains when placed in allopatry with triploid brown trout.

The current study also highlighted that numerous factors can influence social ranking during allopatry, including ploidy status, leading to advantages such as increased access to food, shelter, and reproductive opportunities gained through competitive encounters (Harwood et al., 2002). The higher social rank of diploids may result in displacement of triploids to less favourable areas when stocked into freshwater fisheries leading to reduced opportunities to access food sources. Larger individual size of stocked all-female triploids into freshwater ecosystems may offer a competitive advantage over wild conspecifics. The

competitive superiority of the dominant individual, irrespective of ploidy will be determined by the ability to defend energetically profitable stream positions during allopatry. Previously it has been shown when released from competition, subordinate individuals shift to use more profitable positions (Fausch & White, 1986), however, the foraging capability and habitat utilisation have yet to be conclusively determined in triploid brown trout. Therefore, future studies should ascertain the food preferences of triploid trout post-stocking and whether diet preferences are similar to wild conspecifics.

It can be presumed that triploids may have similar ecological requirements to wild conspecifics and therefore niche overlap may result as similar feeding behaviour is expressed post-stocking into freshwater ecosystems. The use of stable isotopes to characterise differences in feeding between ploidy and the trophic interactions therein would allow a complete characterisation of triploid food sources once released into freshwater fisheries. Recently, stable isotopes have been used to provide an integrated signal of diet over space and time, which makes them very useful in studying population niches (Jackson et al., 2012). In addition, the interaction between triploids and wild conspecifics may change according to season. Limitations to terrestrial invertebrate prey items as a result of lower winter temperature may cause a shift in foraging status. Furthermore, triploids have been shown to have a higher metabolic rate at lower temperature than diploids, which could manifest through increased feeding activity during winter (Atkins & Benfey, 2008). Therefore, field studies should assess whether triploid trout prey upon smaller conspecifics as a results of reduced food availability during winter months. This assessment is critical to assess the impact of the EA stocking policy on natural recruitment within freshwater ecosystems.

In this study, differences between floating and sinking diets were observed whereby dominant individuals acclimated to a floating diet consumed more surface food items than dominant individuals fed a sinking diet when placed into an experimental stream. The

conditioning of trout to floating diets during culture appears to encourage surface-feeding behaviour and could therefore be important to ensuring that triploid trout will rise to a dry-fly post-stock-out. Anecdotal reports from anglers suggest that triploid brown trout are less inclined to surface feeding than diploid brown trout which until now has not been conclusively proven (*pers. comm.* EA, 2010). Results from the current study indicate that surface feeding between ploidy was not significantly different irrespective of diet fed suggesting triploids do rise and consume surface food items. On the other hand, the variation in feeding ability may relate to increased light sensitivity or lower thermal optima, which may therefore promote deeper swimming of triploids in the water column. Variation in feeding ability was not tested in this study due to the depth of the experimental stream however, may also relate to cognitive differences due to changes in brain morphology as suggested in triploid Atlantic salmon (smaller olfactory bulb, larger cerebellum and telencephalon) (Fraser et al., 2012a). Given that size of the cerebellum and telencephalon appear to be linked to foraging strategy and swimming ability (McIntyre & Healy, 1979; Kolm et al., 2009), it could be expected that triploids would be more aggressive and effective feeders. However, evidence to date, including the current study, suggests the opposite (Garner et al. 2008), and therefore reduced foraging ability may be due to other ploidy-dependent differences such as reduced aggression in triploids. Fraser et al. (2012a) also proposed that the increase in size of the triploid cerebellum and telencephalon may not necessarily imply a greater cognitive ability and aggression, as cell numbers are still likely to be less than in diploids, with subsequent effect on neuronal connections.

The value of triploid brown trout to reduce or avoid genetic interactions between farmed and wild conspecifics requires an evaluation of their behaviour and performance within the natural environment. However, to date very few studies have examined the performance of triploids in the natural environment. Dillion et al. (2000) used mixed-sex triploid rainbow

trout to assess the relative return to creel of triploids post-stocking in Idaho, USA. Overall catch returns were not significantly different between ploidy and the time to harvest did not differ between groups. Koenig et al. (2011) suggested fisheries managers should consider stocking all-female rainbow trout (AF3N) in alpine lakes in Idaho, USA. This approach is a low-risk option of maintaining stocks within alpine lakes while minimising the impacts on native stocks. AF3N rainbow trout were recaptured at higher proportions than mixed-sex 3N and diploid 2N rainbow trout through a combination of gill netting and angling effort. High and Meyer (2009) used radio transmitters to track triploid rainbow trout in an Idaho stream and indicated that the average life expectancy was 14.3 days, and at 30 days post-stocking 85% of stocked trout were presumed dead. The higher aggression rates of diploids may be displacing triploids to less productive areas of the stream, which have less access to shelter therefore increasing predation risk. Wagner et al. (2006) compared the growth performance of triploid rainbow trout in three Idaho rivers and found no significant difference in mean weight or feed conversion ratio of either ploidy. However from the lack of significant studies it appears the use of triploids in recreational fisheries needs more investigation especially the ecological interactions with wild conspecifics.

It must be highlighted that mixed-sex triploids were used and that gender could not be determined in the flume study. Males are, however, consistently more aggressive than females even at the juvenile stage in salmonids (Johnsson & Akerman, 1998; Johnsson et al., 2001). In addition, triploidy infers sex-specific differences on reproductive behaviour because steroid production in male triploid fish is significantly greater than in female triploid fish and allows the development of functional testes and secondary sexual characteristics (Schafhauser-Smith and Benfey, 2001). Although spermatogenesis may occur in male triploid fish, random segregation of trivalents results in aneuploid sperm (Benfey & Sutterlin, 1984b; Lincoln & Scott, 1984; Linhart et al, 2006), which fail to produce viable offspring (Lincoln & Scott,

1984; Schafhauser-Smith & Benfey, 2003). For this reason the EA has enforced the legislative policy of only allowing stocking with all-female triploids and not mixed-sex triploid brown trout. Further work is needed to compare gender differences in feeding and behavioural interactions in triploid brown trout to determine whether these differ outside the spawning season. Recently, Fjelldal et al. (2014) indicated that triploid male Atlantic salmon will attempt to spawn with wild conspecifics when in riverine conditions. This study indicated that triploid male Atlantic salmon displayed the full range of secondary sexual characteristics and spawning behaviours of wild males, and stimulated the wild female to spawn in the absence of wild males. It is thus clear that further work is needed to increase the understanding of triploid behavioural interactions post-stock-out into fisheries and their reproductive and behaviour status during the spawning season. Although this work is very difficult to measure in the field, the previous work by Fjelldal et al. (2014) should be used as a model to construct further behavioural work of triploid brown trout interactions with wild conspecifics during the spawning season to ensure wild diploid populations are not impacted unintentionally through such stocking policies. On the other hand, the EA policy is all-female triploid and therefore reproductive interactions would not be expected to occur, however, this is still to be fully elucidated.

3. The effect of temperature on feeding and swimming performances.

The basis of the environmental sensitivity and physiology study (Chapter 5) was to determine the influence of temperature (low or high) on feed intake and whether triploidy affected recovery from exhaustive exercise under these different thermal regimes. The results demonstrated that feed intake profiles were similar at lower temperature (10 °C); however, triploids had a significantly higher feed intake at high temperature (19 °C). These results are contrary to those, which were predicted and generate some interesting questions relating to

the performance of triploids under sub-optimal environmental conditions. Recently, other studies on triploid rainbow trout supported such findings and indicated that triploids feed more, have a greater feed intake and grow quicker than diploids, however, temperature was not a fixed factor within the experimental design of these studies (Cleveland and Weber, 2013; Weber et al., 2014). The results of the current study suggest that triploids may therefore provide freshwater fisheries with a suitable alternative to diploids and higher feeding rates at high temperature, and are thus equally suitable for stocking within a sports fishery as their diploid counterparts

Results of the exercise protocol generated a fascinating insight into the haematological response of diploid and triploid brown trout post-exercise. These data suggest differences between ploidy exist, in line with other studies (Hyndman et al., 2003a, b), however, these differences were not sufficient to impede a full recovery from the simulated "capture" protocol. In addition, the remaining fish from this study were placed in a common-garden experiment and grown-on for a further eight months in tanks. At the end of the trial, body mass was not significantly different between ploidy suggesting little long-term effect of the exercise protocol. The exercise protocol used in our study was longer in duration (10 min) than in previous studies (5 min), therefore, differences between ploidy would have been expected to become apparent over the duration of the exercise. Our results highlight subtle but important differences in blood homeostasis between ploidy within brown trout; however, these results are in contrast to other studies which have reported major differences between ploidy in other salmonid species, indicating that some of the physiological effects of ploidy may be species-and/or environment-specific. For example, Hyndman et al. (2003a) showed that when diploid and triploid brook trout were acclimated to 19 °C and then chased to exhaustion, all diploids survived while 90 % of triploids died. This contrasts greatly with our study and provokes questions regarding differences between studies. One clear difference is

the body mass of the triploid fish used in each study (Hyndman et al., 2003a, ~300 g vs. 80 g in current study) and that greater amounts of lactate are generated in larger fish. Indeed, results of the current study indicate the influence of allometry with larger triploid individuals having more pronounced plasma lactate leading to increased acidosis (~20 mmol / L) than smaller triploids in our study (~6.5 mmol / L). In addition, studies on controlled levels of exercise in rainbow trout showed that larger fish generate proportionally more lactate and protons (lower pH) than smaller fish (Goolish, 1989; Ferguson et al., 1995) suggesting larger fish may suffer greater metabolic stress than smaller fish.

Irrespective of ploidy, in the current study it was demonstrated that brown trout sensitivity of the blood transport system to exercise resulted in changes to both acid-base status and secondary stress responses in brown trout. Post-exercise (e.g. angling stress) the presence of excess protons, i.e. a reduction in blood pH, reduces both haemoglobin-oxygen ($Hb\text{-}O_2$) affinity and O_2-carrying capacity during the Root effect. Although the O_2-carrying capacity of diploid or triploid brown trout was not determined in this study, lower plasma pH of triploids during exhaustive exercise may reduce the affinity of blood for O_2 (Bohr effect) therefore impacting the maximal carrying capacity (Root effect). While in the current study differences in plasma pH were modest between ploidy, other studies suggest that in salmonids even small deviations in pH can have large effects on oxygen carrying capacity of the blood (Pelster & Weber, 1990; Brauner & Randall, 1996; Bernier et al., 2004). In other studies, triploid Atlantic salmon have recovered better than diploids based on the post-exercise oxygen consumption rate (Lijalad and Powell, 2009). Triploid Chinook salmon had reduced arterial O_2 content in comparison to diploids during sustained swimming trials suggesting a reduced factorial scope (Bernier et al. 2004). This concurs with previous work by Graham et al. (1985) who found a reduced $Hb\text{-}O_2$ loading ratio in Atlantic salmon blood. Results of the current exercise study in brown trout have implications for the aquaculture sector within England and

Wales, which will be producing larger triploids to stock into natural environments to support freshwater sport fisheries. Future work should determine the effect of temperature on the stress response of larger triploids to determine whether post-exercise recovery is compromised. The opportunity to evaluate the effects of body mass on haematological parameters was not possible within the current study and would therefore warrant further investigation before we can demonstrate allometry in triploid brown trout. In addition, this study failed to highlight major differences between ploidy previously demonstrated in other studies (Hyndman et al., 2003a) that is likely to be due to the temperatures selected (10 and 19 °C). On the other hand, using temperatures above those chosen within this study may have revealed greater effects of temperature on blood haematology however; using extreme temperature has welfare implications for the experimental animals involved.

Given that triploid salmonids are reported to be more prone to spinal and jaw deformities (Fjelldal & Hansen, 2010; Fraser et al., 2013b; Taylor et al., 2013), which, depending on their severity, may impact swimming (Cotterell & Wardle, 2004; Hansen et al., 2010) and respiratory ability (Lijalad & Powell, 2009), the final aspect of the exercise study evaluated spinal deformity of diploid and triploid brown trout post-exercise. Results of x-ray radiography indicated that there was little difference in the prevalence of skeletal deformity between diploid and triploid brown trout (Appendix III), and that prevalence was extremely low (<1 %), which is in direct contrast to studies in other triploid species (reviewed in Fraser et al., 2012c). This may be due to a combination of factors; firstly, an optimised HP shock has been utilised; secondly, eggs and alevins were reared at low temperature (7 °C) prior to elevating temperature at first-feeding, which has been demonstrated to minimise skeletal and jaw malformation in triploid Atlantic salmon (Fraser et al, 2015); and thirdly, differing environmental pressures on skeletal development, whereby many of the reported malformations manifest themselves in the saltwater environment rather than freshwater. In our

study deformity was principally located within the cranial trunk and caudal trunk areas in triploid trout as opposed to the tail region observed in diploids. The incidence of deformity and its mitigation is thus important from both a farming and welfare perspective. In addition, the prevalence of deformities reduces the aesthetic and commercial value of the fish but could also affect swimming ability and subsequently the fighting qualities of a sport fish (Ornsrud et al., 2004, Powell et al., 2009). However, from this study it appears that deformity prevalence in diploid and triploid brown trout may be less of a concern than in other salmonids, although future work should evaluate different hatchery thermal regimes and dietary influences on the incidence of malformation in farmed triploid brown trout as part of long-term studies.

4. Conclusions

This thesis presents a refined HP shock which delivers 100 % triploid rate in all assessed brown trout, a prerequisite to compliance with freshwater legislation. This research established robust auditing of triploidy through the development of a microsatellite panel to underpin existing verification techniques and ensure compliance with legislation. Also, this work advanced current knowledge on triploid behaviour and potential impact of stocking triploids in freshwater fisheries, how improvements can be made through different feeding techniques and thermal tolerance and the implications for stocking into certain environments highlighting the quality of triploids as a sporting fish. The research and development presented herein support the aquaculture and freshwater fisheries sectors within the United Kingdom.

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PUBLICATIONS AND CONFERENCES

PUBLICATIONS

Preston, A. C., Taylor, J. F., Craig, B., Bozzolla, P., Penman, D., Migaud, H., 2013. Optimisation of triploidy in brown trout (*Salmo trutta* L.). Published in *Aquaculture*. **414**, 160-166.

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CONFERENCE PRESENTATIONS

Preston, A. C., Taylor, J. F., Craig, B., Bozzolla, P., Penman, D., Migaud, H., 2011. Optimisation of triploidy in brown trout (*Salmo trutta* L.). British Trout Association Conference: 28-29th November 2011, Lechlade, England. **Oral presentation**.

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Preston, A. C., Taylor, J. F., P., Penman, Migaud, H., 2012. Optimisation of triploid brown trout (*Salmo trutta L.)* production and assessment of physiological, behavioural and welfare performances. Institute of Fisheries Management Conference: 3-5th October 2012, Edinburgh, Scotland. **Poster presentation**.

Preston, A. C., Taylor, J. F., P., Penman, Migaud, H., 2012. Optimisation of triploid brown trout (*Salmo trutta L.)* production and assessment of physiological, behavioural and welfare performances. World Aquaculture Conference (WAS) European Aquaculture Conference (EAS): 2-5th September 2012, Prague, Czech Republic. **Oral presentation**.

Preston, A. C., Taylor, J. F., P., Penman, Migaud, H., 2012. Optimisation of triploid brown trout (*Salmo trutta L.)* production and assessment of physiological, behavioural and welfare performances. Institute of Aquaculture Postgraduate Research Conference: 24th October 2012, Stirling, Scotland. **Poster presentation.**

Preston, A. C., Taylor, J. F., P., Penman, Migaud, H., 2013. Optimisation of triploid brown trout (*Salmo trutta L.)* production and assessment of physiological, behavioural and welfare performances. Wild Trout Trust Conference: 8-9th June 2013, Dartmoor, England. **Oral Presentation.**

Preston, A. C., Taylor, J. F., P., Penman, Migaud, H., 2013. Optimisation of triploid brown trout (*Salmo trutta L.)* production and assessment of physiological, behavioural and welfare performances. Environment Agency National trout and Grayling Strategy Group AGM: 29 May 2013, Birmingham, England. **Oral Presentation.**

Preston, A. C., Taylor, J. F., P., Penman, Migaud, H., 2013. (1) Triploid induction in salmonids and (2) A review of the verification of ploidy techniques in teleosts. AQUAEXCEL training course 17th -22nd November 2013. Institute of Aquaculture, University of Stirling, Scotland. **Lecturing**.

APPENDIX I

Triploid brown trout survey 2010-2011.

Section 1: General Questions

1. Do you produce all-female triploid brown trout?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	100.0%	2	40.0%	2	57.1%	4
No	0.0%	0	60.0%	3	42.9%	3

2. Do you produce diploid brown trout?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	100.0%	2	40.0%	2	57.1%	4
No	0.0%	0	60.0%	3	42.9%	3

3. Which of the following categories best classifies your facility?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Hatchery	n/a	2	n/a	1	n/a	3
Fishery	n/a	0	n/a	0	n/a	0
Restocking Farm	n/a	2	n/a	5	n/a	7
Table farm	n/a	0	n/a	1	n/a	1

4. How long (years) have you grown all-female triploids brown trout?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Other	100.0%	2	100.0%	4	100.0%	6

5. Do you supply all-female triploids brown trout to other farms?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	50.0%	1	40.0%	2	42.9%	3
No	50.0%	1	60.0%	3	57.1%	4

6. Which of the following water quality parameters are monitored?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Ammonia	n/a	1	n/a	2	n/a	3
Nitrite	n/a	1	n/a	0	n/a	1
Nitrate	n/a	1	n/a	0	n/a	1
CO2	n/a	1	n/a	1	n/a	2
Suspended solids	n/a	1	n/a	2	n/a	3
Alkalinity	n/a	1	n/a	0	n/a	1
Hardness	n/a	0	n/a	0	n/a	0
Temperature	n/a	2	n/a	4	n/a	6
Dissolved oxygen	n/a	1	n/a	4	n/a	5
Flow rate	n/a	2	n/a	1	n/a	3

Section 2: Triploid Induction Questions

7. How is triploid induction achieved?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Hydrostatic pressure shock	100.0%	2	100.0%	3	100.0%	5
Temperature shock	0.0%	0	0.0%	0	0.0%	0

8. What water is used to induce triploidy?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
River	0.0%	0	0.0%	0	0.0%	0
Spring	50.0%	1	33.3%	1	40.0%	2
Borehole	50.0%	1	66.7%	2	60.0%	3

9.a. What water temperature is used to induce triploidy using a hydostatic pressure shock?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
5°C	0.0%	0	0.0%	0	0.0%	0
6°C	0.0%	0	0.0%	0	0.0%	0
7°C	0.0%	0	0.0%	0	0.0%	0
8°C	50.0%	1	0.0%	0	33.3%	1
9°C	0.0%	0	0.0%	0	0.0%	0
10°C	0.0%	0	100.0%	1	33.3%	1
11°C	50.0%	1	0.0%	0	33.3%	1

10. How many minutes post fertilisation (MPF) is the shock applied to fertilised eggs?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
25 MPF	0.0%	0	0.0%	0	0.0%	0
26 MPF	0.0%	0	0.0%	0	0.0%	0
27 MPF	0.0%	0	50.0%	1	25.0%	1
28 MPF	0.0%	0	0.0%	0	0.0%	0
29 MPF	0.0%	0	0.0%	0	0.0%	0
30 MPF	50.0%	1	50.0%	1	50.0%	2
31 MPF	0.0%	0	0.0%	0	0.0%	0
32 MPF	0.0%	0	0.0%	0	0.0%	0
33 MPF	0.0%	0	0.0%	0	0.0%	0
34 MPF	50.0%	1	0.0%	0	25.0%	1
35 MPF	0.0%	0	0.0%	0	0.0%	0
36 MPF	0.0%	0	0.0%	0	0.0%	0

11. What is the duration of the shock (mins)?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
4 mins	0.0%	0	0.0%	0	0.0%	0
5 mins	50.0%	1	0.0%	0	25.0%	1
6 mins	0.0%	0	50.0%	1	25.0%	1
7 mins	50.0%	1	50.0%	1	50.0%	2
8 mins	0.0%	0	0.0%	0	0.0%	0
12. At what stage during the spawning season are triploids produced?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Start	n/a	0	n/a	1	n/a	1
Middle	n/a	2	n/a	2	n/a	4
End	n/a	0	n/a	0	n/a	0

13.a. Is egg quality assessed for triploid induction?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	100.0%	2	100.0%	2	100.0%	4
No	0.0%	0	0.0%	0	0.0%	0
13.b. If so, how is egg quality assessed?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Colour	0.0%	0	0.0%	0	0.0%	0
Feel	0.0%	0	0.0%	0	0.0%	0
Appearance	50.0%	1	50.0%	1	50.0%	2
Ovarian fluid pH	0.0%	0	0.0%	0	0.0%	0
Other	50.0%	1	50.0%	1	50.0%	2

Section 3: Triploid growth performance and behaviour questions

14. How does triploid brown trout growth compare to diploids during production?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Poorer	0.0%	0	33.3%	1	20.0%	1
Equal	0.0%	0	33.3%	1	20.0%	1
Better	50.0%	1	33.3%	1	40.0%	2
Other	50.0%	1	0.0%	0	20.0%	1

15.a. Do triploids exhibit shoaling behaviour and if so when during culture?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	0.0%	0	66.7%	2	40.0%	2
No	50.0%	1	33.3%	1	40.0%	2
Other	50.0%	1	0.0%	0	20.0%	1
Yes	0%	0	50.0%	1	50.0%	1
No	0%	0	50.0%	1	50.0%	1
Same	0%	0	0.0%	0	0.0%	0

16.a. Are triploid brown trout more aggressive than diploids brown trout?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
More aggressive	0.0%	0	0.0%	0	0.0%	0
Less aggressive	50.0%	1	0.0%	0	20.0%	1
No difference in aggression	50.0%	1	100.0%	3	80.0%	4
16.b. If more aggressive how is the aggression exhibited?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Swimming charges	0%	0	0%	0	0%	0
Nipping behaviour	0%	0	0%	0	0%	0
Chasing behaviour	0%	0	0%	0	0%	0

17. Will triploids take a floating pellet?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	100.0%	2	75.0%	3	83.3%	5
No	0.0%	0	0.0%	0	0.0%	0
Other	0.0%	0	25.0%	1	16.7%	1

18.a. Are higher deformities seen in triploids or diploids?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Triploids	n/a	2	n/a	3	n/a	5
Diploids	n/a	0	n/a	0	n/a	0
No difference	n/a	0	n/a	0	n/a	0
Other	n/a	0	n/a	1	n/a	1
18.b. At which life stages are deformities higher?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
alevins	n/a	2	n/a	2	n/a	4
fry	n/a	2	n/a	1	n/a	3
parr	n/a	1	n/a	1	n/a	2
yearling	n/a	1	n/a	1	n/a	2
adult	n/a	1	n/a	1	n/a	2

Section 4: Sex reversal (neomale) production questions

19. Are sex-reversed (neomales) broodfish cultured?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Yes	50.0%	1	100.0%	2	75.0%	3
No	50.0%	1	0.0%	0	25.0%	1

20. What is the concentration of 17á-Methyltestosterone (MT) used for sex re-	Triploid brown trout	All (excluding current survey)	All (including current survey)

versal (mg/kg feed)?						
1-2 mg/kg	0.0%	0	0.0%	0	0.0%	0
3-4 mg/kg	0.0%	0	100.0%	2	66.7%	2
5-6 mg/kg	100.0%	1	0.0%	0	33.3%	1
7-8 mg/kg	0.0%	0	0.0%	0	0.0%	0
>8 mg/kg	0.0%	0	0.0%	0	0.0%	0

Section 5: Triploid production questions

22. Please indicate annual production of all-female triploid brown trout (tonnes)?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
1-5 t	50.0%	1	0.0%	0	20.0%	1
6-10 t	0.0%	0	66.7%	2	40.0%	2
11-15 t	50.0%	1	33.3%	1	40.0%	2
16-20 t	0.0%	0	0.0%	0	0.0%	0
>20 t	0.0%	0	0.0%	0	0.0%	0

23. Are records kept of all triploids produced/supplied?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
yes	100.0%	2	100.0%	3	100.0%	5
no	0.0%	0	0.0%	0	0.0%	0

24. To which areas are all-female triploid brown trout sent?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
England	n/a	2	n/a	2	n/a	4
Avon	n/a	1	n/a	1	n/a	2
Bedfordshire	n/a	0	n/a	0	n/a	0
Berkshire	n/a	1	n/a	1	n/a	2
Bristol	n/a	1	n/a	1	n/a	2
Buckinghamshire	n/a	0	n/a	0	n/a	0

Cambridgeshire	n/a	0	n/a	0	n/a	0
Cheshire	n/a	0	n/a	1	n/a	1
Cleveland	n/a	0	n/a	0	n/a	0
Cornwall	n/a	1	n/a	0	n/a	1
County Durham	n/a	0	n/a	0	n/a	0
Cumbria	n/a	0	n/a	0	n/a	0
Derbyshire	n/a	1	n/a	2	n/a	3
Devon	n/a	1	n/a	1	n/a	2
Dorset	n/a	1	n/a	1	n/a	2
East Riding of York-shire	n/a	0	n/a	0	n/a	0
East Sussex	n/a	0	n/a	1	n/a	1
Essex	n/a	0	n/a	0	n/a	0
Gloucestershire	n/a	1	n/a	0	n/a	1
Greater Manchester	n/a	0	n/a	1	n/a	1
Hampshire	n/a	1	n/a	1	n/a	2
Hereford & Worcester	n/a	0	n/a	0	n/a	0
Hertfordshire	n/a	0	n/a	0	n/a	0
Humberside	n/a	0	n/a	1	n/a	1
Huntingdonshire	n/a	0	n/a	0	n/a	0
Isle of Wight	n/a	0	n/a	0	n/a	0
Kent	n/a	1	n/a	1	n/a	2
Lancashire	n/a	0	n/a	1	n/a	1
Leicestershire	n/a	0	n/a	0	n/a	0
Lincolnshire	n/a	1	n/a	1	n/a	2
London	n/a	0	n/a	0	n/a	0
Merseyside	n/a	0	n/a	0	n/a	0
Middlesex	n/a	1	n/a	1	n/a	2
Norfolk	n/a	1	n/a	0	n/a	1
Northamptonshire	n/a	0	n/a	1	n/a	1
Northumberland	n/a	0	n/a	0	n/a	0
North Yorkshire	n/a	1	n/a	1	n/a	2
Nottinghamshire	n/a	0	n/a	1	n/a	1

Oxfordshire	n/a	0	n/a	0	n/a	0
Shropshire	n/a	0	n/a	1	n/a	1
Somerset	n/a	1	n/a	1	n/a	2
South Yorkshire	n/a	0	n/a	0	n/a	0
Staffordshire	n/a	0	n/a	2	n/a	2
Suffolk	n/a	0	n/a	0	n/a	0
Surrey	n/a	0	n/a	1	n/a	1
Sussex	n/a	0	n/a	1	n/a	1
Tyne & Wear	n/a	0	n/a	0	n/a	0
Warwickshire	n/a	0	n/a	1	n/a	1
West Midlands	n/a	0	n/a	1	n/a	1
West Sussex	n/a	0	n/a	1	n/a	1
West Yorkshire	n/a	1	n/a	0	n/a	1
Wiltshire	n/a	1	n/a	0	n/a	1
Yorkshire	n/a	1	n/a	1	n/a	2
Wales	n/a	1	n/a	0	n/a	1
Brecknockshire	n/a	0	n/a	0	n/a	0
Caernarfonshire	n/a	0	n/a	0	n/a	0
Cardiganshire	n/a	0	n/a	0	n/a	0
Carmarthenshire	n/a	0	n/a	1	n/a	1
Clwyd	n/a	0	n/a	1	n/a	1
Denbighshire	n/a	0	n/a	1	n/a	1
DyfedFlintshire	n/a	0	n/a	0	n/a	0
Glamorgan	n/a	0	n/a	1	n/a	1
Gwent	n/a	0	n/a	1	n/a	1
Gwynedd	n/a	0	n/a	0	n/a	0
Merionethshire	n/a	0	n/a	0	n/a	0
Mid Glamorgan	n/a	0	n/a	0	n/a	0
Monmouthshire	n/a	0	n/a	0	n/a	0
Montgomeryshire	n/a	0	n/a	0	n/a	0
Pembrokeshire	n/a	0	n/a	0	n/a	0
Powys	n/a	0	n/a	1	n/a	1
Radnorshire	n/a	0	n/a	0	n/a	0

South Glamorgan	n/a	0	n/a	0	n/a	0
West Glamorgan	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1

Section 6: Triploid verification questions

25. How is triploidy status verified?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
RBC measurements	n/a	1	n/a	0	n/a	1
Flow Cytometry	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	2	n/a	3

26. Which company is used for triploidy verification?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Other	n/a	1	n/a	1	n/a	2

Section 7: Feed and stocking questions

27. Which feed manufacturer is used during all-female triploid brown trout production?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Skretting	100.0%	2	100.0%	3	100.0%	5
Ewos	0.0%	0	0.0%	0	0.0%	0
Biomar	0.0%	0	0.0%	0	0.0%	0

28. How do you feed all-female triploid brown trout?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Belt feeders	n/a	0	n/a	3	n/a	3
Hand	n/a	2	n/a	3	n/a	5
Demand feeders	n/a	0	n/a	0	n/a	0
Timed hoppers	n/a	0	n/a	0	n/a	0

29.a. What stocking densities (kg/m3) occur of all-female triploids during production and please indicate if additional aeration is used?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
29.b. What stocking densities occur at <5g (kg/m3)	Triploid brown trout		All (excluding current survey)		All (including current survey)	
0-10	0.0%	0	0.0%	0	0.0%	0
10-20	50.0%	1	100.0%	1	66.7%	2
20-30	0.0%	0	0.0%	0	0.0%	0
30-40	50.0%	1	0.0%	0	33.3%	1
29.c. Additional aeration supplied	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Pump and stone	n/a	0	n/a	0	n/a	0
Oxygen	n/a	0	n/a	0	n/a	0
Paddlewheel	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1
29.d. 5-50g -- What stocking densities occur (kg/m3)	Triploid brown trout		All (excluding current survey)		All (including current survey)	
0-10	0.0%	0	50.0%	1	25.0%	1
10-20	50.0%	1	0.0%	0	25.0%	1
20-30	0.0%	0	50.0%	1	25.0%	1
30-40	50.0%	1	0.0%	0	25.0%	1
29.e. 5-50g – Additional aeration supplied	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Pump and stone	n/a	0	n/a	0	n/a	0
Oxygen	n/a	1	n/a	0	n/a	1
Paddlewheel	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1

29.f. 50-100g -- What stocking densities occur (kg/m3)	Triploid brown trout		All (excluding current survey)		All (including current survey)	
0-10	0.0%	0	50.0%	1	25.0%	1
10-20	50.0%	1	0.0%	0	25.0%	1
20-30	50.0%	1	50.0%	1	50.0%	2
30-40	0.0%	0	0.0%	0	0.0%	0
29.h. 50-100g -- Additional aeration supplied	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Pump and stone	n/a	0	n/a	1	n/a	1
Oxygen	n/a	1	n/a	0	n/a	1
Paddlewheel	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1
29.i. 100-200g -- What stocking densities occur (kg/m3)	Triploid brown trout		All (excluding current survey)		All (including current survey)	
0-10	0.0%	0	100.0%	2	50.0%	2
10-20	100.0%	2	0.0%	0	50.0%	2
20-30	0.0%	0	0.0%	0	0.0%	0
30-40	0.0%	0	0.0%	0	0.0%	0
29j. 100-200g -- Additional aeration supplied?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Pump and stone	n/a	0	n/a	1	n/a	1
Oxygen	n/a	1	n/a	0	n/a	1
Paddlewheel	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1
29k. 200-400g -- What stocking densities occur (kg/m3)?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
0-10	0.0%	0	100.0%	2	50.0%	2
10-20	100.0%	2	0.0%	0	50.0%	2
20-30	0.0%	0	0.0%	0	0.0%	0
30-40	0.0%	0	0.0%	0	0.0%	0

29.l. 200-400g -- Additional aeration supplied?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Pump and stone	n/a	0	n/a	1	n/a	1
Oxygen	n/a	1	n/a	0	n/a	1
Paddlewheel	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1
29.m. >400g -- What stocking densities occur (kg/m3)?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
0-10	0.0%	0	100.0%	2	50.0%	2
10-20	100.0%	2	0.0%	0	50.0%	2
20-30	0.0%	0	0.0%	0	0.0%	0
30-40	0.0%	0	0.0%	0	0.0%	0
29.n. >400g – Additional aeration supplied?	Triploid brown trout		All (excluding current survey)		All (including current survey)	
Pump and stone	n/a	0	n/a	1	n/a	1
Oxygen	n/a	1	n/a	0	n/a	1
Paddlewheel	n/a	0	n/a	0	n/a	0
Other	n/a	1	n/a	0	n/a	1

APPENDIX II

A. Panel information of microsatellite loci including, sample number (BT 1-20), number of alleles (1, 2, 2* or 3) observed at each locus, and ploidy of individuals (dip = diploid; trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold. (BT indicates brown trout sample number, ND indicates no data observed).

	Fish ID																			
Panel/Locus	BT-1	BT-2	BT-3	BT-4	BT-5	BT-6	BT-7	BT-8	BT-9	BT-10	BT-11	BT-12	BT-13	BT-14	BT-15	BT-16	BT-17	BT-18	BT-19	BT-20
Ssa410UOS	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	2	1	2	2	ND	2	2	2
Ssa197	2*	2*	2*	2*	2	**3**	2*	2*	1	2*	2*	2*	2	1	2	2	ND	2	2	1
ppStr2	2*	**3**	2*	2*	2*	**3**	21	ND	**3**	2*	2*	2*	2	1	2	2	ND	1	2	2
One102-b	2	2*	**3**	**3**	2*	2*	2*	ND	2*	2*	**3**	2*	2	2	2	2	ND	2	2	2
CA048828	2*	**3**	**3**	**3**	**3**	**3**	**3**	ND	**3**	2*	2*	2*	1	2	1	2	ND	2	2	2
ppStr03	2*	2*	**3**	**3**	**3**	2*	**3**	2*	2*	2*	2*	2*	1	2	2	1	ND	1	2	1
Ssa406UOS	1	2*	**3**	2*	2*	2*	2*	ND	2*	**3**	**3**	2*	2	1	2	2	ND	2	2	2
One 103	2*	2*	**3**	2*	**3**	**3**	2*	ND	2*	2*	2*	2	1	2	2	2	ND	1	1	2
SSa416	1	2*	1	1	2*	1	2*	ND	2*	1	1	1	1	1	2	2	ND	1	2	1
SSaD48	**3**	2*	**3**	2	2	**3**	**3**	ND	**3**	2*	2	**3**	2	2	1	2	ND	2	2	2
Cocl-Lav-4	1	1	1	2	1	2	1	ND	**3**	2*	2	**3**	2	2	1	2	ND	2	2	2
One9uASC	**3**	1	1	2	1	1	2	ND	2	2*	2	2*	2	2	2	1	ND	2	1	2
One102a	1	2*	1	1	1	1	1	ND	1	1	1	1	1	1	1	1	ND	1	1	1
CA054565	1	1	1	1	1	1	1	ND	1	1	1	1	1	1	1	1	ND	1	1	1
CA053293	1	2*	2	2	**3**	**3**	2*	2	1	**3**	2	1	2	2	2	1	ND	2	2	1
CA060177	1	**3**	2*	2*	2*	2*	2*	ND	1	2*	2*	2*	2*	1	2	1	ND	2	2	1
MHC1	2*	2*	2*	2*	2*	**3**	2*	1	2*	2*	2*	2*	2	1	2	1	ND	1	1	1
SSaD71	2*	2	2	2	2	2*	2	2*	2*	2*	**3**	2*	2	2	2	2	ND	2	2	2
SSaTap2A	2*	2	2*	**3**	1	2*	2	2*	1	2	**3**	2*	2	2	1	2	ND	2	2	2
BG93548	2*	2*	2*	1	**3**	1	2*	2*	2	1	2*	2*	1	1	1	4	ND	1	2	2
SSa85	1	1	2	1	1	1	1	2	1	2	2	1	2	1	1	1	ND	1	2	2
No. of Loci w	**3**	**4**	**7**	**5**	**6**	**8**	**4**	**1**	**5**	**3**	**5**	**3**	0	0	0	0	0	0	0	0
No. of Loci w	1	2	3	5	3	1	3	2	2	2	5	1	13	10	13	12	0	12	16	13
No. of Loci w	1	2	3	5	3	1	3	2	2	2	5	1	13	10	13	12	0	12	16	13
Ploidy status	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	dip	dip	dip	dip	dip	dip	dip	dip

B. Panel information of microsatellite loci including, sample number (BT 21-40), number of alleles (1, 2, 2* or 3) observed at each locus, and ploidy of individuals (dip = diploid; trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold. (BT indicates brown trout sample number, ND indicates no data observed).

	Fish ID																			
Panel/Locus	BT-21	BT-22	BT-23	BT-24	BT-25	BT-26	BT-27	BT-28	BT-29	BT-30	BT-31	BT-32	BT-33	BT-34	BT-35	BT-36	BT-37	BT-38	BT-39	BT-40
Ssa410UOS	2	2	1	2	3	**3**	2*	**3**	**3**	**3**	2*	2*	**3**	2*	2*	**3**	2	2	2	2
Ssa197	1	1	1	1	2*	2*	2*	2*	2*	1	2*	2*	1	1	2*	1	2	1	2	2
ppStr2	2	2	1	2	2*	2*	**3**	2*	2*	2*	2	**3**	2*	2*	2*	2*	1	2	2	2
One102-b	2	2	2	2	3	2*	2*	**3**	2*	2*	2*	**3**	**3**	2*	**3**	**3**	2	2	2	2
CA048828	1	1	2	2	3	21	**3**	**3**	**3**	**3**	2*	2*	**3**	2*	**3**	**3**	2	2	2	2
ppStr03	2	2	1	1	2*	2*	2*	2*	2*	2*	2*	1	2*	2*	2*	2*	1	1	2	1
Ssa406UOS	2	2	2	2	3	**3**	2*	2*	2*	2*	**3**	**3**	2	**3**	2*	**3**	2	2	1	2
One 103	2	2	2	2	2*	2*	2*	**3**	1	2	1	2	2*	2*	2*	2*	1	2	2	1
SSa416	1	1	1	2	1	1	2*	1	2*	1	1	1	1	2*	2*	1	1	1	1	1
SSaD48	2	1	2	1	3	**3**	**3**	**3**	2	**3**	**3**	2	**3**	2	**3**	**3**	2	2	2	1
Cocl-Lav-4	2	1	2	1	3	**3**	**3**	**3**	2	**3**	**3**	2	**3**	2	**3**	**3**	2	2	2	1
One9uASC	1	1	1	1	1	1	1	1	1	2	1	2	2	1	2	2	1	2	2	2
One102a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA054565	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA053293	2	2	1	2	2	1	**3**	2	2	2	2	**3**	2	2	2	2	2	2	1	2
CA060177	2	2	1	2	1	1	**3**	2*	2*	1	2*	**3**	1	2*	2*	1	2	2	2	2
MHC1	1	2	1	2	1	2*	2*	2*	**3**	2	2*	2	2	2*	2*	2	1	2	2	2
SSaD71	2	2	1	2	2	2	2	2	2	2*	2*	2*	2*	2	2*	2*	1	2	2	2
SSaTap2A	1	2	2	1	3	2*	1	2*	2*	**3**	**3**	2	2*	2*	2*	**3**	2	2	1	1
BG93548	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SSa85	1	2	2	2	1	2	1	2	2	2	2	**3**	2	1	1	2	2	2	2	1
No. of Loci with 3 alleles	0	0	0	0	**7**	**4**	**6**	**6**	**3**	**5**	**4**	**6**	**5**	**1**	**4**	**7**	0	0	0	0
No. of Loci with 2 alleles	12	12	8	12	2	2	1	3	5	5	3	6	5	4	2	4	11	15	14	11
No. of Loci with 1 alleles	12	12	8	12	2	2	1	3	5	5	3	6	5	4	2	4	11	15	14	11
Ploidy status	dip	dip	dip	dip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	dip	dip	dip	dip

C. Panel information of microsatellite loci including, sample number (BT 41-60), number of alleles (1, 2, 2* or 3) observed at each locus, and ploidy of individuals (dip = diploid; trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold. (BT indicates brown trout sample number, ND indicates no data observed).

	Fish ID																			
Panel/Locus	BT-41	BT-42	BT-43	BT-44	BT-45	BT-46	BT-47	BT-48	BT-49	BT-50	BT-51	BT-52	BT-53	BT-54	BT-55	BT-56	BT-57	BT-58	BT-59	BT-60
Ssa410UOS	2	2	2	2	2	2	2	2	**3**	**3**	**3**	**3**	2*	2*	**3**	**3**	**3**	2*	**3**	**3**
Ssa197	2	2	1	2	2	1	1	2	2*	2*	2*	**3**	1	1	2*	2*	2*	**3**	2*	2*
ppStr2	2	2	1	2	2	2	1	2	**3**	2*	2*	**3**	1	**3**	2*	**3**	**3**	2*	**3**	2*
One102-b	2	2	2	2	2	2	2	2	2	2*	2*	**3**	1	**3**	**3**	2*	2*	**3**	**3**	2*
CA048828	2	1	2	1	2	2	2	2	2	**3**	2*	**3**	1	**3**	**3**	**3**	**3**	2*	**3**	2*
ppStr03	2	2	1	1	1	2	1	1	2*	1	1	2*	2*	2*	2*	2*	**3**	2*	2*	1
Ssa406UOS	1	2	2	2	2	2	2	2	2*	2*	2	2*	2*	**3**	2*	2*	2*	**3**	**3**	**3**
One 103	2	2	2	1	1	2	2	2	1	2*	**3**	2*	**3**	1	**3**	2*	**3**	**3**	**3**	**3**
SSa416	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
SSaD48	1	2	2	2	2	2	1	2	**3**	1	2*	**3**	2	**3**	**3**	2	2	**3**	2	2
Cocl-Lav-4	1	ND	1	1	1	1	1	1	2	1	1	2	2	1	1	1	1	1	1	1
One9uASC	2	2	2	2	1	1	2	2	2	2	1	2	2	2	2	2	1	2	2	2
One102a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA054565	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA053293	2	2	2	2	2	2	1	2	2	2	2	**3**	2	1	2	2	2	2	2	2
CA060177	2	2	2	2	2	2	2	2	2*	1	1	**3**	1	1	2*	2*	1	1	1	2*
MHC1	2	2	2	2	2	2	1	1	2*	2*	1	2	2	1	2*	2*	2*	2	2*	1
SSaD71	2	2	2	2	2	2	2	2	2*	2	2	1	2	2	2*	2*	2	2*	2*	2
SSaTap2A	2	2	1	2	1	2	2	1	**3**	1	2*	**3**	**3**	2*	2*	1	1	2	1	2*
BG93548	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SSa85	2	1	1	2	1	2	2	1	2	1	1	2	1	1	2	1	2	1	2	1
No. of Loci with 3 alleles	0	0	0	0	0	0	0	0	**4**	**2**	**2**	**9**	**2**	**5**	**5**	**3**	**5**	**5**	**6**	**3**
No. of Loci with 2 alleles	14	14	11	13	11	14	10	12	6	3	3	4	6	2	3	3	4	4	4	4
No. of Loci with 1 alleles	14	14	11	13	11	14	10	12	6	3	3	4	6	2	3	3	4	4	4	4
Ploidy status	dip	dip	dip	dip	dip	dip	dip	dip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip	trip

D. Panel information of microsatellite loci including, sample number (BT 61-80), number of alleles (1, 2, 2* or 3) observed at each locus, and ploidy of individuals (dip = diploid; trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold. (BT indicates brown trout sample number, ND indicates no data observed).

	Fish ID																			
Panel/Locus	BT-61	BT-62	BT-63	BT-64	BT-65	BT-66	BT-67	BT-68	BT-69	BT-70	BT-71	BT-72	BT-73	BT-74	BT-75	BT-76	BT-77	BT-78	BT-79	BT-80
Ssa410UOS	2	2	2	2	2	2	2	1	2	2	2	2	**3**	2*	**3**	**3**	**3**	**3**	**3**	**3**
Ssa197	2	2	2	1	2	2	2	2	1	2	2	1	1	2*	1	2*	1	2*	**3**	2*
ppStr2		2	1	1	2	1	2	2	2	2	2	2	1	2*	**3**	2*	2*	ND	**3**	**3**
One102-b		2	2	2	2	2	2	1	2	2	2	1	**3**	2*	2*	**3**	**3**	ND	**3**	2*
CA048828		2	2	2	2	1	2	1	2	2	2	2	2	**3**	2*	**3**	2*	ND	**3**	2*
ppStr03	2	1	2	2	1	1	2	1	1	1	2	2	**3**	2*	1	2*	2*	**3**	**3**	2*
Ssa406UOS		2	2	1	2	2	2	2	2	2	2	2	2*	**3**	2	2*	2*	ND	**3**	2*
One 103	2	2	2	2	2	2	2	2	2	2	2	2	2*	**3**	**3**	2*	2*	ND	**3**	2
SSa416	1	1	1	1	1	1	1	1	1	2	1	2	1		1	1	2	ND	1	1
SSaD48	ND	2	2	2	2	2	1	1	2	2	2	2	2*	**3**	2	**3**	**3**	ND	**3**	2*
Cocl-Lav-4	2	1	ND	1	1	1	1	ND	1	1	1	2	2	1	1	2	1	ND	2	2
One9uASC	1	2	1	2	2	2	2	1	1	2	2	2	1	1	2	2	2	ND	2	1
One102a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ND	1	1
CA054565	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ND	1	1
CA053293	2	2	2	1	1	2	1	2	2	2	1	1	2	2	1	**3**	**3**	ND	**3**	3
CA060177	1	2	2	2	1	2	1	2	2	2	2	2	1	1	2*	2*	**3**	1	1	2*
MHC1	2	2	2	1	1	1	2	2	2	2	2	2	2	1	2*	1	2*	1	2	2
SSaD71	2	2	1	2	2	2	2	1	2	2	2	2	2*	2*	2*	2	2*	2	2*	**3**
SSaTap2A	2	2	2	2	2	2	2	2	2	2	2	1	**3**	2*	2	2*	2*	**3**	2*	2*
BG93548	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SSa85	2	2	1	1	2	1	1	2	2	2	2	2	1	1	2	1	1	ND	2	2
No. of Loci with 3 alleles	0	0	0	0	0	0	0	0	0	0	0	0	**4**	**4**	**3**	**5**	**5**	**3**	**10**	**4**
No. of Loci with 2 alleles	10	15	12	10	12	11	12	9	13	16	15	14	4	1	5	3	2	1	4	4
No. of Loci with 1 alleles	10	15	12	10	12	11	12	9	13	16	15	14	4	1	5	3	2	1	4	4
Ploidy status	dip	dip	dip	dip	dip	dip	dip	dip	dip	dip	dip	dip	trip	trip	trip	trip	trip	trip	trip	trip

E. Panel information of microsatellite loci including, sample number (BT 81-96), number of alleles (1, 2, 2* or 3) observed at each locus, and ploidy of individuals (dip = diploid; trip = triploid). All loci with three alleles and the total number of loci having three alleles are indicated in bold. (BT indicates brown trout sample number, ND indicates no data observed).

	Fish ID															
Panel/Locus	BT-81	BT-82	BT-83	BT-84	BT-85	BT-86	BT-87	BT-88	BT-89	BT-90	BT-91	BT-92	BT-93	BT-94	BT-95	BT-96
Ssa410UOS	**3**	**3**	**3**	2*	2	2	2	2	2	1	2	2	2	2	1	2
Ssa197	2*	2*	2*	2*	2	2	1	2	2	2	1	1	2	2	2	2
ppStr2	2*	**3**	**3**	2*	1	2	2	2	1	2	1	1	2	2	2	2
One102-b	2*	2*	2*	**3**	2	1	2	2	2	2	2	2	2	2	2	2
CA048828	2*	**3**	2	2	2	1	2	2	1	2	2	1	2	2	2	2
ppStr03	2*	2*	2*	2*	2	2	2	1	2	2	2	1	2	2	2	1
Ssa406UOS	1	2*	2*	2	1	2	2	2	2	2	2	2	2	2	2	1
One 103	**3**	2*	2*	2*	2	2	2	1	1	1	2	2	2	2	1	2
SSa416	1	1	1	2*	1	1	2	1		1	1	1	1	1	1	1
SSaD48	**3**	2	**3**	**3**	2	2	2	2	2	2	2	2	1	2	2	2
Cocl-Lav-4	1	2	1	1	1	2	1	1	2	2	1	1	1	1	1	2
One9uASC	2	1	1	2	2	2	2	2	1	1	2	2	2	1	1	2
One102a	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1
CA054565	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CA053293	2	2	2	2	2	1	1	2	2	2	2	1	2	2	2	2
CA060177	2*	1	2*	1	2	2	2	2	2	2	2	1	2	1	2	2
MHC1	2*	1	1	**3**	2	1	2	2	1	1	1	1	1	2	2	2
SSaD71	2*	2*	2	2*	2	2	2	2	2	2	2	1	2	1	2	2
SSaTap2A	1	1	2*	2*	1	2	2	2	1	2	2	2	2	2	2	2
BG93548	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SSa85	2	2	1	1	1	1	2	1	1	1	2	1	2	1	2	2
No. of Loci with 3 alleles	**3**	**3**	**3**	**3**	0	0	0	0	0	0	0	0	0	0	0	0
No. of Loci with 2 alleles	3	4	3	4	12	13	15	13	10	12	13	7	14	12	13	15
No. of Loci with 1 alleles	3	4	3	4	12	13	15	13	10	12	13	7	14	12	13	15
Ploidy status	trip	trip	trip	trip	dip	dip	dip	dip	dip	dip	dip	dip	dip	dip	dip	dip

APPENDIX III

Typical diploid and triploid brown trout radiographs.