A COMPARATIVE STUDY OF GENE EXPRESSION IN WILD AND DOMESTICATED ATLANTIC SALMON (*SALMO SALAR L.*)

A thesis submitted to the University of Stirling for the degree of Doctor of Philosophy

By

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Declaration

I declare that this thesis has been compiled by myself, and is the result of my own investigation. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

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Abstract

Atlantic salmon (*Salmo salar* L.) has been domesticated since the 1960s and has undergone over 10 generations of artificial selection for economically important traits. As a result, domesticated salmon have diverged with respect to a number of phenotypic, genotypic and behavioural traits from their wild counterparts. Since the selection pressures that are present in the wild differ greatly from the ones that shape salmon under culture conditions, domesticated salmon stocks are considered to be maladapted to natural conditions. Despite strict regulations, insoluble issues pertaining to large-scale cage rearing of farmed fish mean that there is a continuous presence of farm escapees in the wild. Gene flow from escapees has been perceived as a factor in the decline of wild populations, suggested to occur through disruption of local adaptation. This study aims to improve understanding of the genetic differences between wild and domesticated stocks by comparing the transcriptomes of Figgjo (wild) and Mowi (domesticated) strains. A series of common garden experiments have been performed, utilizing pure and reciprocal hybrid crosses of the wild and domesticated stocks, reared under two different conditions and sampled at four time points and three distinct life stages (embryo, sac-fry and feeding fry). Microarray interrogations were performed employing a 44K custom microarray design to identify genes and gene pathways that are differentially expressed between the stocks. KEGG-based functional analyses have been implemented using different gene set enrichment packages, and dominance and additive parameters were calculated from normalized expression values to predict the mode of heritability of the genes identified as differentially expressed between stocks.

Most biological functions represented in wild and domesticated crosses were consistent across life stages and environments. The transcriptomic differences detected between stocks in multiple developmental stages likely reflected adaptations to selection pressures differing between natural and aquaculture environments. Down-

regulated *environmental information processing* and *immune* and *nervous system* functions in domesticated vs. wild fish may be due to local adaptation to captivity. These included reduced information acquisition and processing systems, altered stress responsiveness and changes in feeding behaviour. In line with the resource allocation theory of production trait animals, reduced immune function was coupled with increased expression of growth and development related pathways in domesticated salmon, compared to wild counterparts. Although there is support for this trade-off in all life-stages, resource allocation showed a shift over time; possibly reflecting variation in the utilization of energy sources during the transition from endogenous to exogenous feeding. Differences in cell communication and signalling pathways between wild and domesticated stocks, associated with organogenesis during the embryo stage, reflect sampling time and are indicative of altered organ development in response to domestication.

Stress responses common across stocks included the down-regulation of *cellular processes,* including *cell cycle and meiosis*, and *genetic information processing*, such as *replication and repair*, *transcription* and *translation* pathways, probably reflecting the reallocation of energy resources away from growth and towards the restoration of homeostasis. Moreover, the mobilization of energy to cover the increased demands of maintaining homeostasis was indicated by the up-regulation of some *metabolic pathways*, mostly involved in *energy*, *lipid* and *carbohydrate metabolism* in response to stress. The analysis also revealed cross-specific stress responses, including indicators of a non-additive stress response in hybrid crosses.

Most differentially expressed transcripts exhibited additive (31-59%) or maternal dominant (19-33%) inheritance patterns, although maternal over-dominance (23-26%) was also significant in the embryo stage. The mode of heritability of some immune transcripts was suggestive of maternal environmental influence having been affected by aquaculture.

This study has demonstrated that biological functions affected by domestication include those associated with allocation of resources, involve reduction of information acquisition and processing systems and may lead to loss of local adaptation to wild conditions. Since such changes may affect key systems, such as immunity and responsiveness to stress, they can potentially have serious negative consequences under natural conditions. Transcriptomic differences observed between wild and domesticated stocks primarily exhibited additive and maternal dominant inheritance modes. Since gene-flow from farmed fish can be frequent and primarily concerns farmed females, this suggests that introgression due to repeated large scale escape events has the capacity to significantly erode local adaptation.

Table of Contents

List of tables

Table 4.2 Proportions of the differentially expressed genes displaying various inheritance patterns. Based on a heritability analysis of the differentially expressed genes and a comparison of the inheritance patterns to a previous study conducted in sac and feeding fry. For explanation of the various categories see the materials and methods section.

Table 5.1 Pathways found to be differentially expressed between wild and domesticated stocks under control and stress conditions by both gage and romer packages. The direction of change shown describes the expression of the pathway in the domesticated fish relative to wild counterparts. The terms “2D” and “Mixed” are used to describe pathways in which genes showed bidirectional change. “Genes” refers to the number of genes included in the gene set test.

Table 5.2 Pathways found to be differentially expressed between control and stress conditions in pure wild and domesticated stocks by both gage and romer packages. The direction of change shown describes the expression of the pathway in the stressed fish relative to the control state. The terms “2D” and “Mixed” are used to describe pathways in which genes showed bidirectional change. “Genes” refers to the number of genes included in the gene set test.

Table 5.3 Pathways found to be differentially expressed between control and stress conditions in reciprocal hybrids by both gage and romer packages. The direction of change shown describes the expression of the pathway under stress condition relative to control condition. The terms “2D” and “Mixed” are used to describe pathways in which genes showed bidirectional change. “Genes” refers to the number of genes included in the gene set test.

Table 5.4 Proportions of the differentially expressed genes displaying various inheritance patterns in the reciprocal hybrids relative to the expression of pure crosses under control and stress conditions.

Appendix

List of figures

Figure 3.2 and 3.3: Hierarchical clustering of the essential genes of the significant pathways for sac (2) and feeding fry (3). Colour coding is based on normalised intensity values

Figure 3.4: Visual representation of heritability of annotated transcripts differentially expressed between experimental groups based on 1-way ANOVA (10% FDR). Error bars show the standard deviation between replicate arrays. Nine over-dominant, one dominant and one recessive transcript were excluded from the graph for easier visualisation.

Figure 4.1 A schematic representation of the experimental design.

Figure 4.2 Key genes of the perturbed pathways. Differentially expressed genes (T-test $p \leq 0.05$) between wild and domesticated embryos and identified as essential for the pathways perturbed between pure stocks (Table 4. 1). Genes are plotted according to $\log_2$ fold change (domesticated vs wild) and $-\log_{10}$ *p*-value (T-test), and color-coded by biological function.

Figure 4.3 Up-regulated differentially expressed transcripts. Hierarchical clustering of the expression profiles of unique transcripts up-regulated in domesticated embryos compared to wild embryos.

Figure 4.4 Down-regulated differentially expressed transcripts. Hierarchical clustering of the expression profiles of unique transcripts down-regulated in domesticated embryos compared to wild embryos.

Figure 4.5 Heritability predictions of the differentially expressed genes between stocks for the two hybrid stocks. DEG: differentially expressed gene, WD: wild♀ × domesticated♂, DW: domesticated♀ × wild♂

Figure 4.6 The number of transcripts differentially expressed between stocks and their inheritance pattern. Differences observed between hybrid and pure crosses are

categorized as influenced by maternal, paternal or parental effects (see Methods for details). The number of differentially expressed transcripts identified between hybrid crosses is also shown.

Figure 5.1 A 3-D representation of the PCA performed on all transcripts that passed quality filtering. Samples are colour coded by the experimental factors.

Figure 5.2 A representation of the number of differentially expressed transcripts based on a 2-way ANOVA. **A.** Transcriptomic differences arising through variation between all crosses (WxW, WxD, DxW, DxD) conditions (stress and control) and the interaction of these two factors. The top numbers reflect statistics for all crosses including the hybrids, whereas the bottom numbers were generated by limiting the 2-way ANOVA to pure crosses only. **B.** The common and unique differences in cross-specific expression with and without consideration of reciprocal hybrids. **C** The common and unique differences arising from exposure to stress vs control conditions and detected with and without consideration of hybrids.

Figure 5.3 Visual representation of heritability of genes differentially expressed between crosses in control (graphs on top) and stress (graphs on bottom) states. Heritability was plotted for both reciprocal hybrids; W♀ x D♂ (on the left) and D♀ x W♂ (on the right). $\alpha > 0$ / $\alpha < 0$ is characteristics to genes that are down/up regulated in domesticated compared to wild fish and $-0.5 < \delta/\alpha < 0.5$ corresponds to additivity, $-1.5 < \delta/\alpha < -0.5$ to wild dominance, $0.5 < \delta/\alpha < 1.5$ to domesticated dominance, and if δ/α falls out of the interval −1.5-1.5, then over-dominance of the expression of the transcripts studied.

Figure 5.4 Hierarchical clustering of the normalised expression values of the genes that were identified as wild (over)dominant in the W♀ x D♂ hybrids, and additive/wild dominant in the D♀ x W♂ hybrids under stress conditions.

Figure 6.1 A comparison of the differentially expressed transcripts between wild and domesticated pure crosses identified by T-tests (corrected $p<0.1$) in the various life stages.

Figure 6.2 Hierarchical clustering of the fold changes of the transcripts that were differentially expressed between wild and domesticated pure crosses in at least three life stages.

Figure 6.3 A comparison of up and down regulated pathways (1d) between wild and domesticated pure crosses identified by *gage*, *roast* and *romer*.

Figure 6.4 A comparison of differentially expressed pathways whose genes showed bidirectional change (2d/Mixed) between wild and domesticated pure crosses according to *gage*, *romer* and *roast* functions.

Figure 6.5 A. A comparison of the differentially expressed transcripts identified between the crosses in the various life stages by 1-way ANOVA (corrected $p<0.1$). B. Differentially expressed transcripts under parental influence. C. Differentially expressed transcripts exhibiting maternal effects. D. Differentially expressed transcripts exhibiting paternal effects

Figure 6.6-6.10 A comparison of the heritability of the differentially expressed genes identified in at least two life stages. It is calculated for both reciprocal hybrids and grouped according to functions.

Figure 6.11-6.15 A comparison of the additive parameters of the differentially expressed genes identified in at least two life stages and their representation by functional groups.

Chapter 1 - General Introduction

1.1 Background of the species

Atlantic salmon (*Salmo salar* Linnaeus, 1758) is classed as a ray-finned fish (*Actinopterygii*), in the order of Salmons (*Salmoniformes*) and family of Salmonids (*Salmonidae*) (Froese and Pauly, 2015). The Salmonidae family purportedly evolved from a common ancestor following a genome duplication event that occurred between 25 and 100 million years ago (Allendorf and Thorgaard, 1984; Ohno, 1970). It consists of three subfamilies: whitefish and ciscos (*Coregoninae*); graylings (*Thymallinae*); and trout, salmon and charr (*Salmoninae*) (Koop and Davidson, 2008) (Figure 1.1).

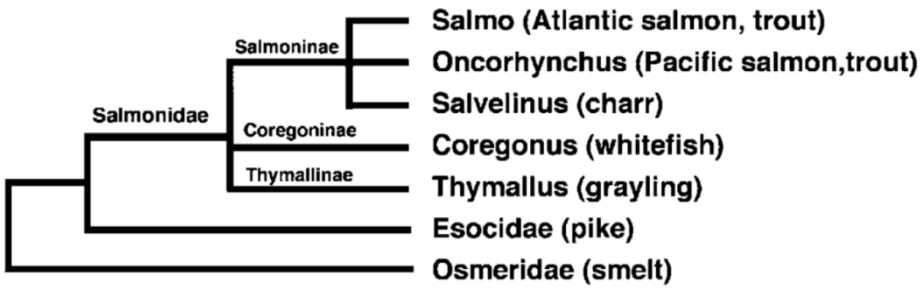


Figure 1.1 General relationships of the major salmonid and closest sister groups (Koop and Davidson, 2008).

The species is primarily anadromous, with a strong tendency to return to natal areas to spawn. However, maturation in freshwater is possible, a relatively few non-anadromous populations being known to exist. The general life cycle (Figure 1.2) is initiated in fresh water, as eggs deposited by the female in the gravel, called redds, are fertilised by male(s). Hatchlings called alevins or sac fry remain in the gravel nourished by their yolk-sac until that is absorbed and swim up/first feeding occurs. The feeding fry enters parr stage with the acquisition of vertical “parr” marks and small red dots on the sides of the body. Smoltification is a physiological transformation that prepares the fish for the shift from fresh to salt water. Following this process, smolts migrate to sea

to complete their oceanic feeding migration before returning to their home rivers to reproduce (Verspoor et al., 2007).

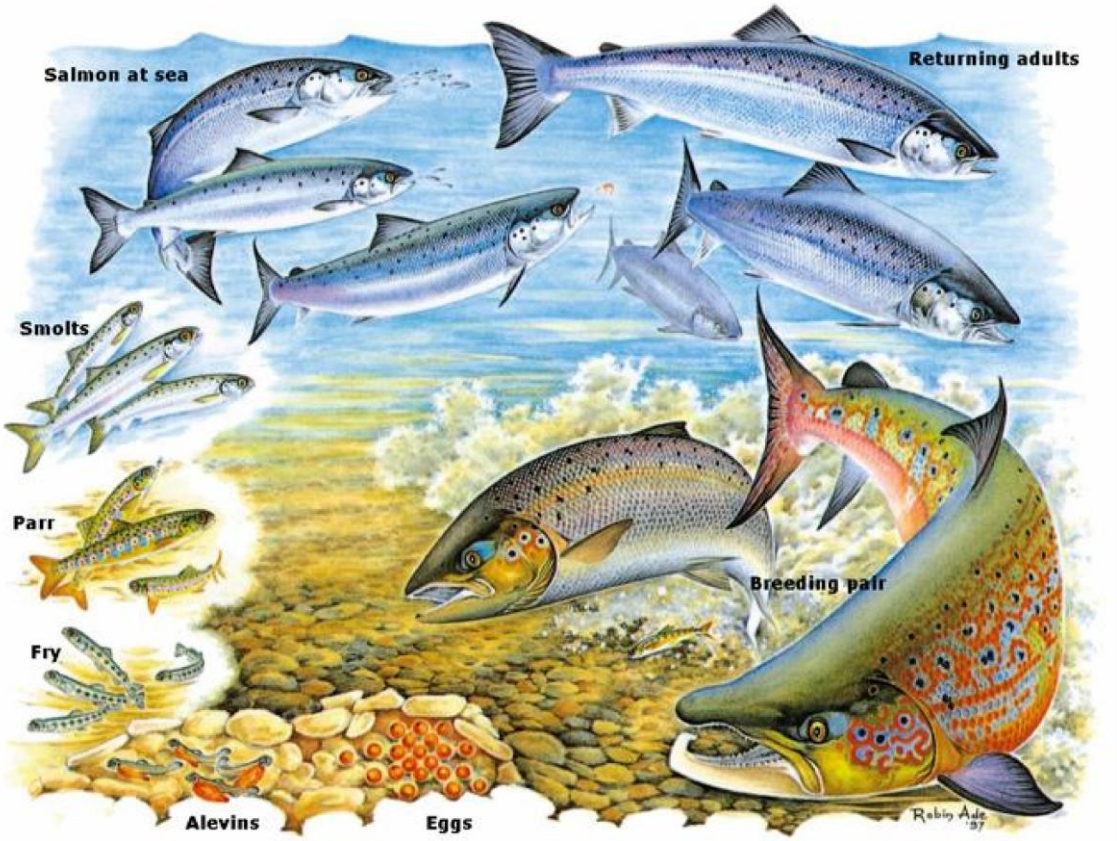


Figure 1.2 Generalised life-cycle of the Atlantic (Atlantic Salmon Trust, image by R. Ade).

Although the species has a complex, tightly regulated life cycle and the ability to adapt to diverse environments, in terms of temperature and salinity depending on their developmental stages, its biogeographic range has been reduced and many populations have been declining. The current distribution of native Atlantic salmon is temperate and subarctic regions of the North Atlantic from northern Portugal (~42˚N) to northwest Russia (~68˚N) in the NE Atlantic and from New England (~41˚N) to northern Quebec (~59˚N) in the NW Atlantic. It is currently estimated to occur in around 2550 rivers of the North Atlantic area (NASCO, 2015). Canada, Norway and Scotland are homes to the vast majority of healthy habitats and approximately 75% of all known salmon rearing rivers (Figure 1.3) (NASCO, 2015; WWF, 2001). The main

causes of the decline are considered to be over-exploitation, climate change and human-induced habitat alteration. The latter includes pollution, activities that alter river flow rate, or present obstacles to migration, as well as aquaculture related activities, such as farmed fish mediated diseases and escaped farmed fish interacting with wild populations (OSPAR Commission, 2010; WWF, 2001).

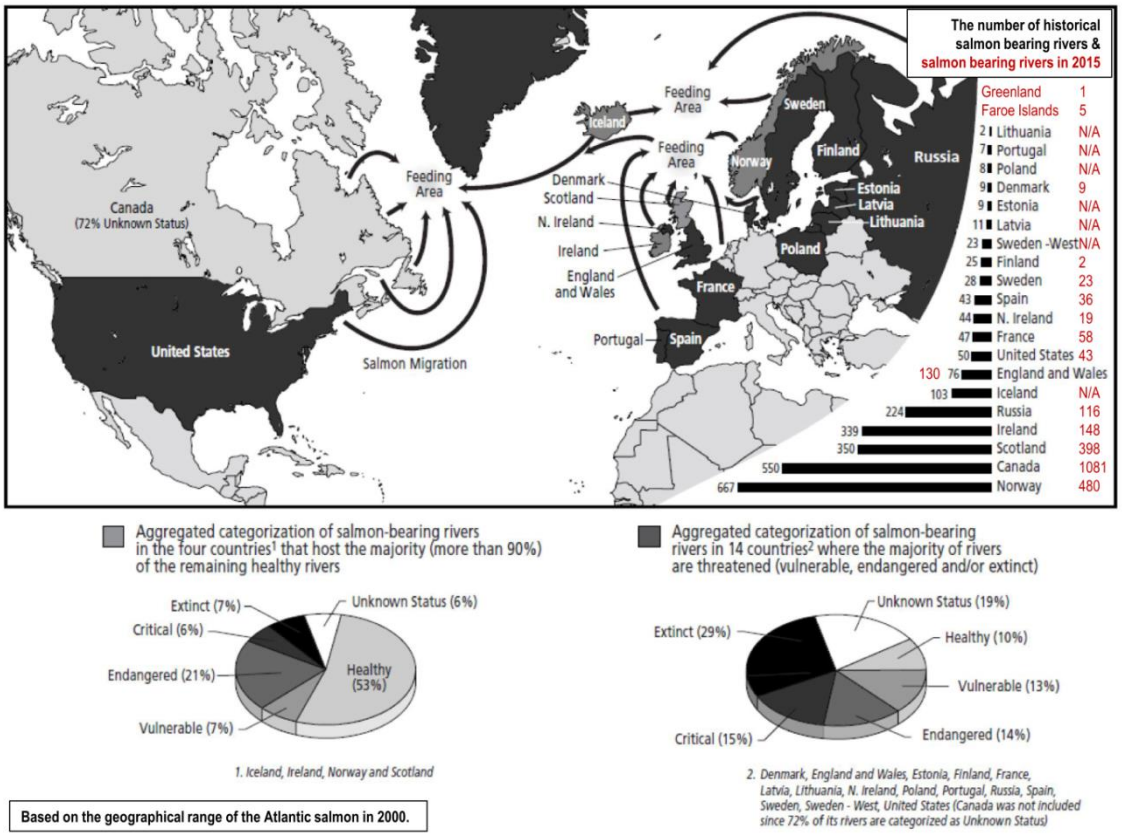


Figure 1.3 Geographic range, health status and global migration routes of Atlantic salmon in 2000 (WWF 2001). The original figure has been modified by adding salmon bearing rivers in 2015 (shown in red), according to the Atlantic Salmon Rivers Database (NASCO, 2015).

Wild Atlantic salmon are both culturally significant and economically important as a food source and in relation to sport-fishing and ecotourism. In addition to its significance to nature, local culture and the general public, the species is an asset to the salmon fishing industry and related businesses. In the North Atlantic region, the gross value of the catch by net and trap fisheries is estimated to €7 million annually, whereas anglers spend approximately €500 million per year. In addition, the global

value of the Atlantic salmon farming industry is worth over €10 billion (Marine Harvest, 2015). The Atlantic salmon further benefit the economy through the jobs created around related businesses. These include fishery owners selling exclusive fishing rights, fishmongers, smokeries, tackle manufacturers and distributers, people working in tourism or in the aquaculture industry (NASCO, 2015).

As a result of its high impact, Atlantic salmon is widely studied. Areas of research include; its biology, life histories, population dynamics, biogeography, phylogenetic relationships, physiology and nutrition and domestication (Davidson et al., 2010). In addition to national schemes, major international efforts are being made to better understand and conserve the species. For example, the North Atlantic Salmon Conservation Organization (NASCO) was established by an inter-governmental Convention in 1984. It aims to conserve, restore, enhance and manage wild Atlantic salmon (NASCO, 2015). Although the decline of wild salmon populations has been correlated with the rapid expansion of the aquaculture industry (Ford and Myers, 2008), this is misleading. Decline in numbers of some populations have been observed as early as the late 1800s (Chaput, 2012). The impact of the industry on wild stocks is not all negative, as their investment in research also benefits the species. For example, the International Cooperation to Sequence the Atlantic Salmon Genome (ICSASG) was established in 2009 concentrating efforts and funding from public and private member organizations and aquaculture industries from Canada, Chile and Norway to sequence the genome of Atlantic salmon (Davidson et al., 2010). Five years later a reference sequence was published on 12th June 2014, although improvements are still on-going (ICSASG, 2014). The Atlantic salmon is a sentinel species, a cultural icon in many coastal communities world-wide, the focus of one of the highest profile recreational fisheries and the basis for one of the World's largest aquaculture industries (OSPAR Commission, 2010). The declining numbers reported for many Atlantic salmon populations worldwide necessitates a better scientific understanding of

this species to provide improved management guidelines. Considering its migratory nature, long-term sustainability of Atlantic salmon requires an international collaboration prioritizing the interest of the species.

1.2 Aquaculture and related issues

Atlantic salmon was first cultured in freshwater in the 19th century in the UK for stocking purposes (FAO, 2015). Sea cage culture was initiated in Norway in the 1960s for commercial purposes, by Mowi a/s in Bergen and Grøntvedt Brothers in Hitra (Gjedrem et al., 1991). The successful captive sea water rearing of Atlantic salmon prompted development of selection programs, standardized rearing cycles (Figure 1.4), and its culture in other countries, leading to the rapid expansion of the industry.

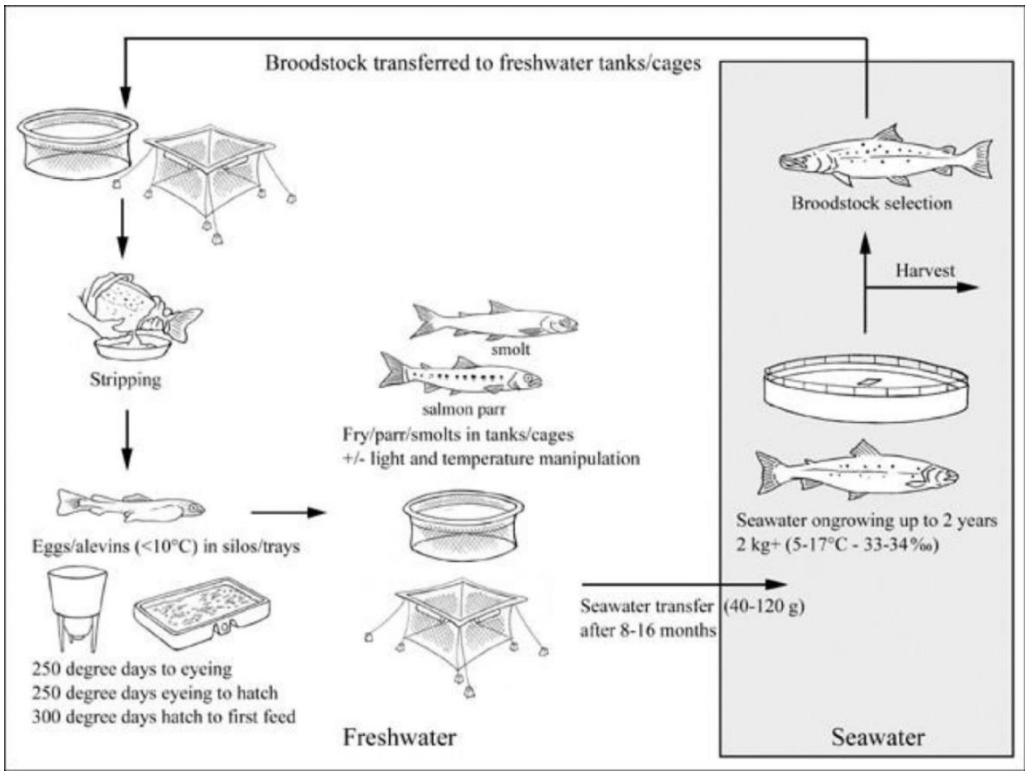


Figure 1.4 The production cycle of Atlantic salmon. Source (FAO, 2015)

Since 2012 the global production has exceeded 2M tonnes annually (Figure 1.5). Scotland, Ireland, the Faroe Islands, Canada, the North East of USA, Chile and Australia (Tasmania) are now major producers (Table 1), with smaller industries in

New Zealand, France and Spain (FAO, 2015). Breeding approaches, starting with the mass phenotypic selection employed by Atlantic salmon farmers in the pioneering phase of the industry, have advanced over time, with complex family-based breeding programs, similar to those successfully employed in terrestrial farming, becoming the norm. The first targeted trait, body weight, was of immediate commercial interest and perceived to be influenced by a large genetic component. As quantitative genetics data became available for other economically important traits, such as late maturation, disease resistance and flesh quality, these were incorporated into breeding programs (Gjedrem, 2010). The process of gaining an understanding of the genetic bases underlying the phenotypic variation of complex production related traits is still ongoing (Tsai et al., 2015).

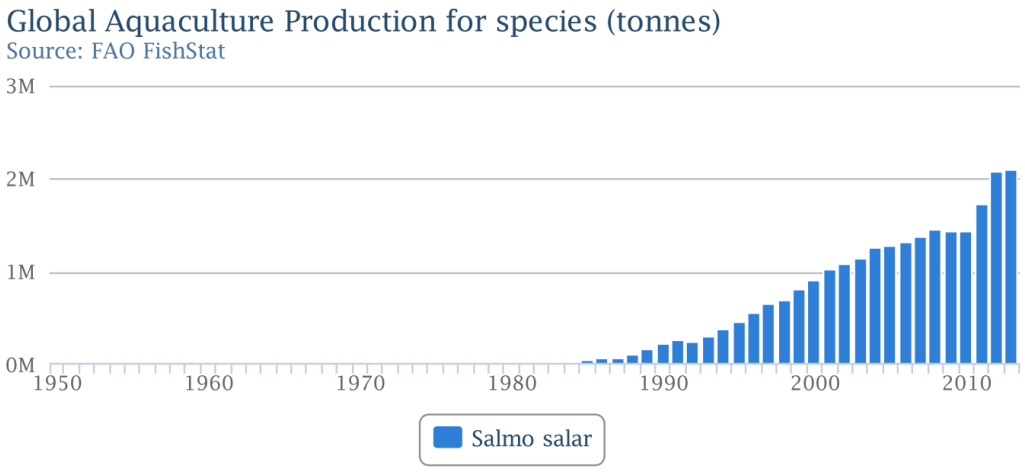


Figure 1.5 The global production of Atlantic salmon until 2013. Source (FAO, 2015)

WW Atlantic salmon supply						
(1000 tones WFE)	2011	2012	2013	2014E	2015E	2016E
Norway	1,006	1,183	1,144	1,204	1,204	1,260
UK	155	159	158	155	160	160
Faroe Islands	56	70	72	77	82	80
Ireland	16	19	14	17	17	18
Europe	**1,233**	**1,431**	**1,387**	**1,453**	**1,463**	**1,518**
Annual growth	*8%*	*16%*	*-3%*	*5%*	*1%*	*4%*
Chile	221	364	470	595	581	590
Canada	110	137	115	123	132	135
USA	18	20	20	20	20	20
Australia	36	37	38	38	38	38
Others	2	9	11	11	11	11
Total Other	**387**	**565**	**654**	**786**	**781**	**794**
Annual growth	*29%*	*46%*	*16%*	*20%*	*-1%*	*2%*
Total global supply	**1,620**	**1,996**	**2,041**	**2,239**	**2,244**	**2,312**
Supply growth%	*12%*	*23%*	*2%*	*10%*	*0%*	*3%*

Source: Kontali and Nordea Markets

Table 1.1 The global supply of Atlantic salmon from 2011-2013, and estimates from 2014-2016.

As production of farmed Atlantic salmon has increased, so has its impact on the environment. The main concerns are related to the sea cage rearing period, specifically the potential for genetic introgression of farmed salmon into wild populations, negative impact of salmon lice and viral diseases on wild salmonid populations, and local and regional impact of nutrients and organic loads. The potential impact of these issues was addressed by a large scale risk assessment carried out in Norway (Taranger et al., 2015), by the world's largest producer of Atlantic salmon. The findings indicated that 25% of the salmon farms affected by sea lice had a moderate to high likelihood of causing significant mortality of wild Atlantic salmon smolts. In addition, during viral outbreaks, viruses are extensively present in areas surrounding the sea cages; however these have low prevalence in wild populations (Taranger et al., 2015). In Norway, coastal aquaculture is controlled by several laws and regulations, including an act regarding the environmental risk assessments of fish farms, that was reinforced in June 2009 (Lundebye, 2013). As a confirmation of the success of these regulations, by 2013 the ecological impact of sea cages has been reduced, since 87% of the assessed fish farms had moderate-to-high ecological conditions under the sea cages and risk of eutrophication and organic over loading beyond the production area of the farm was also considered low (Taranger et al.,

2015). However, the problem of containment of farmed fish and concerns regarding potential genetic interactions between farmed escapees and wild fish still persist today (Saunders, 1991; Taranger et al., 2015).

Containment of farmed fish remains an issue, as despite strict regulations, thousands to hundreds of thousands of Atlantic salmon are reported annually to escape from sea cages. Indeed, the actual number of farmed escapees is believed to be much higher, due to significant underreporting by the industry (Glover, 2010; Glover et al., 2008; Zhang et al., 2013). While the majority of escaped fish 'disappear' in the sea (Hansen, 2006; Skilbrei, 2013, 2010a, 2010b), nevertheless a significant number have been observed returning to some rivers inhabited by wild populations throughout their geographic range (Fiske et al., 2006; Gausen and Moen, 1991; Gudjonsson, 1991; Morris et al., 2008; Volpe et al., 2014; Webb et al., 1993; Youngson et al., 1997). Moreover, despite the reduced reproductive success of farmed salmon compared to wild fish under natural conditions (Fleming et al., 2000, 1996), farmed escapees are known to successfully reproduce (Clifford et al., 1998; Crozier, 1993; Glover et al., 2012; Skaala et al., 2006). The potential introgression between stocks is a concern, as gene flow from farmed fish is likely to disrupt locally adapted genetic structuring of wild populations (Bourret et al., 2011; Glover et al., 2012, 2013; Skaala et al., 2006). Indeed, it has been demonstrated in a number of studies that the offspring of farmed salmon display lower survival under natural conditions than its wild counterpart (Fleming et al., 2000; McGinnity et al., 2003, 1997; Skaala et al., 2012).

1.3 The biology behind domestication

"The power of Selection, whether exercised by man, or brought into play under nature through the struggle for existence and the consequent survival of the fittest (Spencer, 1864), absolutely depends on the variability of organic beings." (Darwin, 1875a)

Plant and animal domestication has been practiced for centuries and it has changed the course of civilization in multiple ways. It triggered a shift from the hunter-gatherer life style to a more predictable producing one. In addition to ready supply of food, it revolutionised clothing, provided transport, workforce and companionship that have profoundly changed people's lives (Diamond, 2002). It is difficult to give a universal definition of domestication or to generalize its effects, due to the wide range of species involved and the various purposes of domestication. Price (1984) gathered many important aspects of domestication that I believe describe domestication well. According to this definition, domestication is an accelerated evolutionary process, where the major selective pressure is towards phenotypes that benefit humans. In addition to artificial selection for desired traits, natural selection in captivity, the absence of natural selection pressure, inbreeding and genetic drift may also influence domesticated populations. Hence, domestication is achieved and maintained through a combination of genetic changes that occur over generations and environmentally induced developmental events that reoccur during subsequent generations (Price, 1984). For a long time, scientists have been trying to understand the nature and effects of domestication and several theories exist that attempt to provide explanations. Darwin recognised that domestication is a form of accelerated evolution through artificial selection (Darwin, 1875a), Price and King (Price and King, 1968; Price, 1984) noted that this process has a genetic and an environmental component, however the molecular mechanisms underpinning domestication and evolution are yet to be fully understood (Jensen, 2015). Although Darwin speculated that adaptation to captivity and stress resistance is key to the process of domestication (Darwin, 1875a), actual evidence has come from an extensive experiment involving foxes (*Vulpes vulpes*) that have displayed a range of common behavioural and physical features characterising domesticated vertebrates, despite only being selected for docility (Belyaev, 1969; Trut et al., 2009). According to a theory formulated by Wilkins and colleagues, these common features that are most pronounced in mammals but apply

to most domesticated vertebrates, stem from mild neural crest cell deficits occurring during embryonic development of these animals. Since embryonic neural crest tissue gives rise to several essential cell lineages, it has the capacity to explain a wide range of specific features that appear in many domesticated animals; including reduction of acute fear and long term stress, through the reduced size and function of the adrenal gland (Wilkins et al., 2014). Similarly, in order to reduce stress associated with captivity, and reliance on humans to meet nutritional and shelter needs, reduced environmental awareness has also been proposed as a consequence of domestication. This could be achieved through the decline of information acquisition and transmission systems, such as sensory organs and synaptic activity (Hemmer, 1990). Stress resistance associated with human presence and captivity, seems plausible as a common feature of domesticated animals, as this condition is shared across species regardless of the purpose of domestication. On the other hand, synaptic activity may or may not have adaptive significance during domestication, depending on its context. For example, animals that are bred to provide companionship to humans, have different selection pressures acting on certain cognitive abilities, than those whose fate is to become livestock (Li et al., 2014). It has also been proposed that domestication is the product of heterochrony, *i.e*. changes in developmental rates and / or timing, induced by thyroid hormone altered oxidation reaction and metabolism rates (Crockford, 2006, 2004, 2003). Thyroid hormones, supplied by the maternal thyroid gland in early life stages, are known to be essential for all stages of normal embryonic development, including neural crest development. In addition to mediating the development of specific morphological traits, thyroid hormones also control stress response (Crockford, 2004).

Universal hypotheses aiming to identify the common roots of domestication, like the *neural crest development* or the *thyroid hormone* theories, both support Belyaev's findings (Belyaev, 1969; Trut et al., 2009), according to which stress resistance is core

to domestication. In fact Crockford goes as far as suggesting that in many cases it is key to the process of speciation, through pre-dispositioning certain individuals to split of the ancestral population and become founders of new populations (Crockford, 2004). A universal aspect of domestication is that it involves interaction with humans and so it makes sense that when looking for common processes of domestication across species, response to this particular range of stimuli is an essential part of that. Due to the large variety of species under domestication and its wide range of purpose, finding common ground is limited. However, when one's interest is not to find universal trends but to find the effects of domestication that apply to more confined units, such as all farm animals or all farmed fish in particular, one will likely to find more commonalities. Indeed, in addition to employing domestication as a model for accelerated evolution (Darwin, 1875a), domestication is often studied to improve the welfare of domesticated animals (Dawkins, 1980), improve their production (Rendel, 1974) and to assess the environmental risks associated with them (e.g. Crosetti 2007), where studies are often simplified to single species or even explored at population level.

In addition to captive breeding and human interactions, different species of livestock share a range of other aspects of their lives. These include increased selection pressure for production related traits under culture condition, since the best performing individuals are more likely to be selected for broodstock. This involves, performing well at high stocking densities; increased feed intake and efficiency in metabolising commercial feed and investing it into meat, milk, egg or wool production for example. According to resource allocation theory, since resources that are available to a given individual are limited, when investment is increased in one trait, a trade-off with another, perhaps momentarily, less important trait will have to occur. Such trade-offs have been proposed between increased production related traits and the high energy demanding immune system (Rauw, 2012).

Compared to other farmed species, Atlantic salmon has been domesticated only relatively recently (Gjedrem et al., 1991). Since it is *reared* for multiple purposes, it is important to differentiate *farmed salmon* from that of *hatchery-reared* and *sea ranched salmon*. Farmed salmon that have undergone several generations of domestication selection are well adapted to aquaculture conditions, show enhanced performance for commercially important traits, and are phenotypically, behaviourally and genetically diverged from wild populations. On the other hand, hatchery-reared and sea ranched salmon has not been artificially selected through breeding programs, but they too are reared under hatchery conditions until released into the wild for stocking purposes at fry, parr, smolt or post-smolt stages (Thorstad et al., 2008). Although hatchery rearing is often a key component of conservation programs, even short term hatchery rearing can induce changes leading to poor performance of these individuals in the wild (Stringwell et al., 2014). Despite the absence of artificial selection pressures for improved production traits, hatchery reared fish faces selection associated with the captive environment, such as the presence of humans, high stocking densities, tank environment, as well as the relaxation of selection pressures that are present in the wild, including predator-avoidance or foraging behaviour. As a result, hatchery-rearing may reduce stress responsiveness (Naslund et al., 2013) and predator awareness (Alvarez et al., 2003) and increase risk-taking behaviour (Roberts et al., 2011), aggression (Blanchet et al., 2008) and growth (Saikkonen et al., 2011) of wild salmonids. To decrease these environmental effects and increase post release survival of hatchery reared fish, minimizing the time spent in captivity and enrichment of the hatchery environment are recommended (Roberts et al., 2014).

The above mentioned differences have been reported for domesticated and wild Atlantic salmon; namely reduce stress responsiveness (Solberg et al., 2013a) and predator awareness (Einum and Fleming, 1997; Aimee Lee S. Houde et al., 2009) and increased aggressiveness (Einum and Fleming, 1997; Fleming and Einum, 1997; A. L.

S. Houde et al., 2009) of domesticated fish compared to wild fish. In addition, due to the effects of artificial selection, domesticated Atlantic salmon display greatly increased growth rates under farming conditions (Glover et al., 2009; Solberg et al., 2013a, 2012; Thodesen et al., 1999), delayed maturation (Gjedrem, 2000), reduced genetic diversity in highly polymorphic genetic markers at the population level (Norris et al., 1999; Skaala et al., 2004) compared to its wild counterparts. Increased disease resistance has also been suggested as a result of Atlantic salmon domestication (Gross, 1998).For example, a strain selected for increased resistance for the infectious pancreatic necrosis virus (IPNV) showed lower mortality in a challenge test, than wild salmon(Gjedrem and Baranski, 2009). Moreover, altered gene-transcription patterns have also been reported in response to domestication, involving energy metabolism, transcription regulation, protein synthesis, immunity, muscle function and digestion(Roberge et al., 2008, 2006).

That offspring of wild salmon show higher fitness *cf*. offspring of hatchery fish (Araki and Schmid, 2010; Araki et al., 2008) and display increased survival compared to the offspring of farmed salmon under natural conditions (Fleming et al., 2000; McGinnity et al., 2003, 1997; Skaala et al., 2012) has been attributed to different selection pressures acting in nature compared to those in hatchery and aquaculture environments. Moreover, important behavioural, life history and morphological Atlantic salmon traits (Dylan J Fraser et al., 2010) show additive genetic variation, and gene expression of divergent salmonid populations also display mainly additive inheritance patterns (Bougas et al., 2010; Debes et al., 2012; Nolte et al., 2009). Maternal effects are also known to be common in salmonids and are mainly associated with egg and nest quality (Green, 2008). Egg and alevin size and survival are also maternally influenced (Einum and Fleming, 2000, 1999; Houde et al., 2011; Skaala et al., 2012).

1.4 Transcriptomics; developments, rationale, advantages and limitations

Due to the sheer size of the salmon aquaculture industry and its predominant use of sea cage culture technologies, potential introgression of farmed fish genes into wild populations, due to inadvertent escapes, is a concern. As a result, a number of technologies have been harnessed in attempts to differentiate between wild and farmed salmon and to assess the genetic consequences of the interactions between them. Predominant has been the application of molecular genetic markers, such as microsatellite loci / short tandem repeats (STRs). These have been used in studies to trace escapees back to their farm of origin (Glover, 2010), and to follow the fate of farmed salmon in wild populations (Bourret et al., 2011; Glover et al., 2012; Skaala et al., 2006; Zhang et al., 2013). With cost-effective and practical platform developments, studies employing informative single nucleotide polymorphisms (SNPs) have been increasing. In addition to looking at differentiation between farmed and wild Atlantic salmon and measurement of gene flow between stocks (Bourret et al., 2011; Karlsson et al., 2011), the much larger numbers of SNP markers that can be routinely screened allow broader scale studies, e.g. identifying footprints of domestication (Mäkinen et al., 2014).

In addition to genomic technologies, the effects of domestication and the consequences of interactions between wild and farmed Atlantic salmon have been investigated using transcriptomic approaches, specifically gene expression profiling. Broad-scale studies have used high throughput technologies such as microarray analysis (Roberge et al., 2008, 2006) and, much more recently, RNA sequencing (Debes et al., 2012; White et al., 2013). Gene expression microarray analysis, the most commonly used method to explore global transcriptional changes, allows for the interrogation of the mRNA expression levels of thousands of genes simultaneously, with relatively high specificity, thereby providing a snapshot in time of global gene

expression. Specific gene expression microarrays for salmonid research have been developed since 2004, with most being designed primarily for commercially important species, namely Atlantic salmon and / or rainbow trout (*Oncorhynchus mykiss*). Key resources for gene expression work in salmon, i.e. tissue –specific cDNA libraries and expressed sequence tag data, were largely generated in the early 2000s. Three major initiatives provided most of these resources; the Canadian funded GRASP project, the EU funded SALGENE project and the Norwegian funded Salmon Genome Project. Early microarrays were cDNA based, with PCR amplicons (*c*. 200-2000 bp in length) derived from cDNA libraries, being pin-spotted onto glass slides (Koop et al., 2008; Rise et al., 2004; Taggart et al., 2008; von Schalburg et al., 2005). A major improvement in technology was the switch to using shorter synthetic oligonucleotides, in place of variable length cDNA amplicons. In the case of the latter, most of the sequence for spotted probes, though available for hybridisation was essentially unknown, only 5' and / or 3' reads being documented. Oligonucleotide probes, on the other hand, are of fully known sequence, standard size, and therefore have much more predictable and consistent hybridisation characteristics. Again the earliest formats were low density, pin printed microarrays, with later commercial platforms (e.g. Affymetrix, Agilent, NimbleGen) using *in situ* means of probe generation (e.g. inkjet and photolithographic technologies) to produce much higher density microarrays (up to 1 M probes per array). Some commercial platforms offer the flexibility of custom design of microarrays from sequence data alone, removing the requirement for access to physical cDNA libraries. The specificity in design of oligonucleotide arrays has proven to be particularly valuable in reducing cross-hybridization and allowing expression patterns of duplicate genes to be more confidently distinguished. This is particularly pertinent to salmonid studies, where duplicate genes, derived from the relatively recent salmonid-specific whole genome duplication event, may differ in DNA sequence by as little as 3-4% (Koop and Davidson, 2008). The first published salmonid oligonucleotide microarray was a trial 5K spotted design (von Schalburg et al., 2008), but this was

soon superseded by a commercial (Agilent) 44K probe array for Atlantic salmon, together with a number of custom-designed arrays, again based on the Agilent platform. For example, Krasnov and colleagues developed an oligo microarray focusing on immunologically important genes (Krasnov et al., 2011), while other more generally applicable designs were also developed by specific laboratories (e.g. (Jantzen et al., 2011). Researchers at the University of Stirling designed a custom salmonid microarray that has been used in an extensive range of studies. This array, designed primarily in 2009, with some later minor enhancements, is based on Agilent's 44K format. It is primarily an Atlantic salmon resource with *c*. 34.5 K features from Atlantic salmon coding sequences together with a further *c*. 9K features from rainbow trout sequences, the latter being selected where no homologs appeared to be available within *S. salar* databases. The features are printed singly. The microarray is broad scope, reflecting the wide range of cDNA sequence data available at the time. This design has been used for infection studies (Herath et al., 2013; Morais et al., 2012; Pooley et al., 2013) and nutritional trials (De Santis et al., 2015b; Glencross et al., 2015; Martinez-Rubio et al., 2012; Morais et al., 2012).

The rationale behind using transcriptomics to investigate evolution, including domestication, comes from the idea that gene regulation governs evolution of anatomy, physiology and behaviour. This pertains to a theory based on the observation that the small degree of molecular divergence present at the protein level of humans and chimpanzees is not sufficient to account for the vast anatomical and behavioural differences between the species (King and Wilson, 1975). The notion that evolutionary change in anatomy is primarily based on changes in the mechanisms controlling gene expression is now widely accepted, and through the emergence of high throughput technologies a greater emphasis is being placed on comparative studies of gene expression, regulation, and development (Carroll, 2005). Indeed, the genetic theory of morphological evolution states that form evolves largely by altering

the expression of functionally conserved proteins (Carroll, 2008). High throughput transcriptomics technologies, including microarrays allow the simultaneous comparison of the expression patterns of thousands of genes across samples. Through the associations made between specific traits and gene expression alterations, they provide clues for gene function, as well as a better understanding of gene regulation at the systems level (Clarke and Zhu, 2006). Based on the overly simplified central dogma (Crick, 1970), DNA is transcribed to RNA and then translated to protein. When studying the transcriptome by gene expression analysis, it is mRNA abundance that is being assessed (Figure 1.6) (Ritchie et al., 2015).

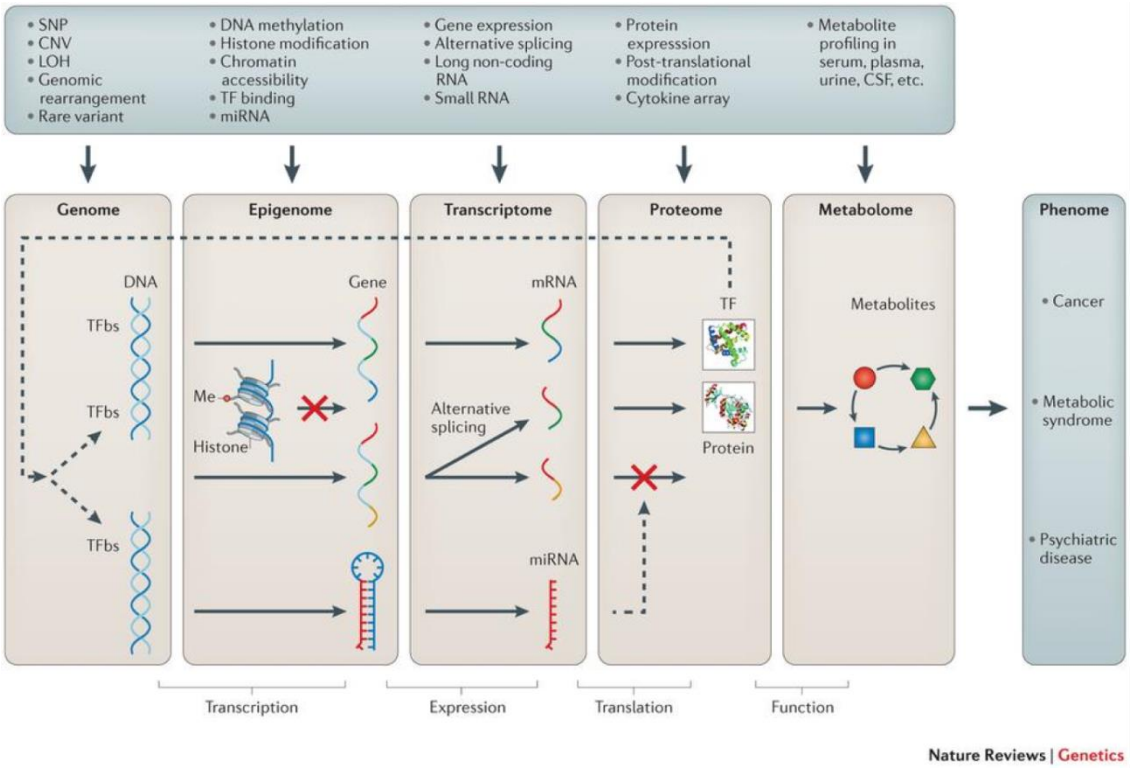


Figure 1.6 A schematic representation of where information is being captured for transcriptomics studies within the flow of genetic information (Ritchie et al., 2015).

In fact, microarrays are designed to compare the relative mRNA transcript abundance reflecting active transcription, transcript stability, and transcript degradation (Clarke and Zhu, 2006). It is important to note that although mRNA levels are often used as a proxy to predict protein abundance, regulatory steps that occur downstream of mRNA

synthesis may affect protein yield and activity (Feder and Walser, 2005). Post-transcriptional regulators, such as mRNA translation efficiency, turnover kinetics, small and large non-coding RNAs influence the overall level of protein produced from an mRNA (Figure 1.7)(Geisler and Coller, 2013; Keene, 2007). Nonetheless, broad scale microarray analysis provides a powerful tool to explore potential gene transcription / regulatory consequences of hybridisation between wild and domesticated salmon.

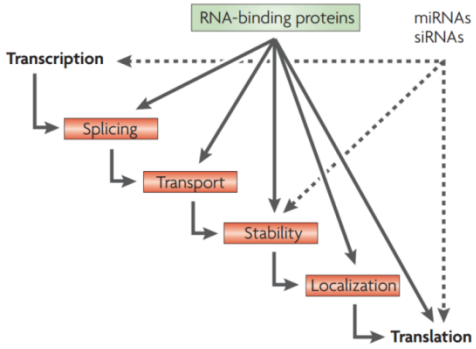


Figure 1.7 Processes downstream of mRNA synthesis that occur between transcription and translation and may affect protein activity of the cell. Post-transcriptional regulations may involve RNA-binding proteins and small non-coding RNAs, such as miRNAs, microRNAs; siRNAs, small interfering RNAs (Keene, 2007).

1.5 Microarray analysis to study the domestication of Atlantic salmon

Microarray analysis has been used to compare the transcriptomes of wild and domesticated salmonid populations, including brook charr (*Salvelinus fontinalis*) (Bougas et al., 2010; Sauvage et al., 2010), rainbow trout (*Oncorhynchus mykiss*)(Tymchuk et al., 2009; White et al., 2013) and Atlantic salmon (Debes et al., 2012; Normandeau et al., 2009; Roberge et al., 2008, 2006). Work conducted by Roberge and co-workers, had elements in common with the research described in the present thesis, since it involved the same species and also examined mRNAs derived from whole individuals, rather than from specific tissues. A further similarity is the target life stage. Both studies focused on early life stages, however, while Roberge and colleagues sampled at initial swim-up phase, samplings undertaken throughout the current thesis were specifically timed to avoid such transitional event life stages. Since oligo microarrays designed for salmonids only became publicly available from 2008 (von Schalburg et al., 2008), the experiments carried out by Roberge and colleagues utilized cDNA microarrays. The work published in 2006 and 2008 used

3.5K and 16K designs available from the GRASP and cGRASP projects respectively. On the other hand, the current study is based on a 44K oligo microarray design and as such it benefits from higher resolution, specificity, capacity, and improved annotation. Further unique aspects of the study reported here are sampling at various early life stages and under environmental conditions and using reciprocal hybrids to elucidate the modes of heritability governing the expression of the transcriptomic differences identified between the crosses. The current study also took advantage of the improved annotation tools that are increasingly available for non-model organisms and the rapidly advancing analysis methods that support the interpretation of high throughput data. The most important such development employed in this study is the use of up-to-date functional annotation through the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (**http://www.genome.jp/tools/kaas/**) and its incorporation into gene set enrichment analyses.

Instead of solely relying on statistical differences of individual genes between experimental groups, gene set analysis integrates knowledge of biological function, typically that of molecular pathways, and examines the expression of putatively co-regulated genes. Differential expression identified by gene set tests is supported by many genes, providing a powerful tool to detect small, but consistent changes; trends in gene expression that gene-wise tests may not be sensitive enough to detect. At the same time gene set analysis simplifies interpretation of the data by incorporating functional information and therefore focuses the attention to biologically meaningful processes. Moreover, gene set analyses are valuable tools to compare gene expression patterns from different studies, platforms, even species (Luo et al., 2009; Wu et al., 2010). Considering that transcriptomic differences between wild and domesticated Atlantic salmon are generally small in terms of fold changes when whole individuals are studied (Roberge et al., 2008, 2006), a robust and sensitive method such as gene set test was particularly valuable for this study. Furthermore, it allowed

for the meaningful interpretation and comparisons of the differences detected between crosses and across life stages and environments.

1.6 Aims and approaches of the study

This PhD study was funded as part of project INTERACT, a strategic institute program run by Institute of Marine Research, Bergen and funded by the Norwegian Research Council.

The overarching objective of the research presented in this thesis was to better characterise differences in transcriptomic profiles of early stages of selected families of wild and domesticated origin salmon and to gain some perspective on the potential impact of introgression between these forms. The specific aims of the work described in the PhD thesis were:

1. To compare transcriptome profiles of wild and farmed Atlantic salmon at different early life-stages and under different environmental conditions
2. To identify genes and gene pathways that are differentially expressed between strains of contrasting origins
3. To gain a better understanding of the evolutionary forces acting on genomes adapted for wild and domestic environments

The principal experimental approach was to rear domesticated, wild and hybrid families in common environments, thereby allowing comparative analyses of transcriptomic states, with minimal uncontrolled environmental influence. The study utilized the long established domesticated Atlantic salmon strain, Mowi, maintained by Marine Harvest at Tveitevåg, Norway. Since the late 1960s (i.e. > 10 generations) the strain has been selected, based on measured phenotypes, for growth, late maturation and fillet quality. From 1999 these and additional traits have been further improved through family selection programmes (Glover et al. 2009). The wild broodstock used in this study originated from the Figgjo River population in the south west of Norway, a

stock which comprises small to medium sized, 1-2 sea-winter returners. Both wild and domesticated fish were stripped and pure wild, pure domesticated and hybrid crosses were established. Fish were reared under identical conditions in IMR's Matre fish station. This study focused on exploring transcriptional differences between stocks in early life stages (Figure 1.8).

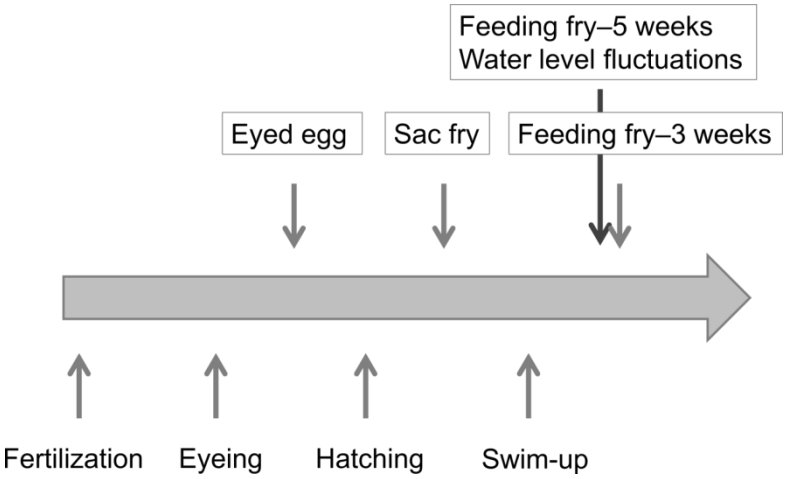


Figure 1.8 A visual representation of the sampling points in relation to major developmental events of the Atlantic salmon

Since the domesticated stock used here diverges early in growth from its wild counterpart (Glover et al., 2009; Solberg et al., 2013a), and body size has been linked to developmental stage in fish, focusing on early life stages ensured that the wild and domesticated stocks were as close as practically possible to developmental synchronisation at the times of sampling (White et al., 2013). Moreover, samplings were timed to avoid transitional event life stages, such as hatching and swim up, when major changes in gene expression occur due to entering a different developmental stage (Jantzen et al., 2011). Transcriptional profiles were detected using a well validated 44K oligo nucleotide microarray design that allowed a more in-depth, and potentially more accurate assessment of transcriptional differences between domesticated and wild salmon than was undertaken in earlier studies.

Chapter 2 - Overview of the experimental chapters

Atlantic salmon have been domesticated for over ten generations leading to the genetic divergence of farmed stocks from wild counterparts. Since the selection pressures that act upon cultured and natural populations are very different, the adaptations that favour a domesticated life-history are rarely advantageous in the wild. A large number of farmed fish escape from fish farms annually, leading to concerns that farmed escapees have the potential to alter the genetic composition of wild populations and thereby disrupt local adaptation. Thus, elucidating the genetic differences and interactions between domesticated and wild salmon is essential to the maintenance of healthy wild stocks and achieve sustainability of the aquaculture industry.

This study is comprised of a series of microarray experiments conducted on the offspring of the long-established farmed Atlantic salmon strain, called Mowi, and members of a wild population from the Figgjo River in Norway. It is aimed to gain a better understanding of the genetic differences between domesticated and wild Atlantic salmon through the comparison of the transcriptome of pure and hybrid stocks that have been reared under identical hatchery conditions. The objective of this chapter is to provide a short summary of the experiments conducted, and to explain how they are linked, in an attempt to provide a more complete picture.

2.1 Overview of chapter 3

In this chapter a preliminary experiment is described, in which the transcriptomes of wild, domesticated and hybrid stocks (domesticated dam x wild sire) were compared at yolk sac absorption and 5 weeks post first-feeding fry stages. Although some of the transcriptional differences detected overlapped between sampling points, the results highlighted the importance of studying various life stages. Compared to the wild population, the Mowi strain displayed up-regulation in *mRNA translation*-related

pathways and down regulation in *nervous* and *immune* system -related pathways in the sac fry, whereas marked up-regulation of *digestive* and *endocrine* activities, *carbohydrate*, *energy*, *amino acid* and *lipid metabolism* and down-regulation of *environmental information processing* and *immune system* pathways were evident in the feeding fry. Differentially regulated pathways that were common between the two life stages generally belonged to *environmental information processing* and *immune system* functional groups. In addition, indications of strong maternal effects were found, reinforcing the importance of including reciprocal hybrids in further experiments in order to distinguish between parental and stock origin effects.

Overall, this research confirmed previous studies in concluding that widespread detectable differences exist between gene expression profiles of fish of domesticated and wild origin. In addition, it found that many of the affected pathways are life-stage specific. The data supports the view that the genetic architecture of the strains highly influences the differential expression of genes between wild and domesticated fish.

2.2 Overview of chapter 4

This experimental chapter compares the transcriptomes of wild and domesticated Atlantic salmon embryos. In addition to exploring a different early life-stage, reciprocal hybrid families are incorporated into the experimental designs in order to dissect parental effects from the effects of domestication.

The most significantly enriched functional groups identified were those involved in *cellular signaling* and the *immune system*. These functional groups, although often represented by different pathways, were also highlighted in the previous study of yolk-sac and feeding fry (Chapter 3). The *mRNA translation* pathways *ribosome* and *RNA transport* were also found to be up regulated in domesticated embryos, this being consistent with results obtained for sac fry. In addition the *focal adhesion* and *gap junction* pathways, relating to *cell communication*, and *cell adhesion molecules*

seemed to be unique differences, affecting only the embryo stage. Examination of heritability indicated strong maternal effects, and in addition to the relevance of additivity and maternal dominance, was suggestive of a higher level of over-dominance compared to later life stages.

Cell signaling and *communication* pathways appeared to be of importance during embryonic development, such as their involvement in organogenesis and thus may be particularly relevant for the embryonic life stage. In addition, the *cellular signaling* pathways have a role in responding to external stimuli including stress, and it is plausible that they have been altered during domestication. Given that the process of domestication involves the provision of an artificial rearing environment, changes in these as well as *immune pathways* are not unexpected. The increase of *mRNA translation* observed in the studied domesticated stock and its relation to protein synthesis may be the product of positive selection for growth in breeding programs. Use of reciprocal hybrids has enabled the determination of whether dominant effects are largely attributable to maternal influence or domestication, and served to highlight the importance of maternal effects. In addition to the relevance of additivity, the data revealed the prominence of over-dominance in the studied embryo samples, this being higher than that observed in sac-fry and exogenous feeders.

2.3 Overview of chapter 5

In this chapter the transcriptomes of the offspring of wild and domesticated Atlantic salmon, inclusive of reciprocal crosses, were compared under standard hatchery environment conditions and in response to an applied stressor. Differences between wild and domesticated crosses were largely consistent under control and stress conditions and included down-regulation of *environmental information processing*, *immune* and *nervous system* KEGG pathways and up-regulation of *genetic information processing*, *carbohydrate metabolism, lipid metabolism* and *digestive* and *endocrine system* pathways in the domesticated fish relative to their wild counterparts, likely

reflective of different selection pressures acting in wild and cultured populations. Major stress responsive functions were also shared between crosses and included down regulation of *cellular processes* and *genetic information processing* and up-regulation of some metabolic pathways, *lipid* and *energy* in particular. These responses may be indicative of mobilization and reallocation of energy in response to stress.

Reciprocal hybrids were again employed to identify the modes of heritability that govern transcriptomic differences between stocks. Additivity and maternal dominance accounted for approximately 42% and 25% respectively, of all differences under control conditions for both hybrids. The mode of inheritance of the genes differentially expressed between stocks under stress was less consistent between reciprocal hybrids, potentially reflecting maternal environmental effects.

2.4 Overview of chapter 6

Microarray experiments described from Chapter 3-5 compared the transcriptomes of the Mowi (domesticated) and Figgjo (wild) Atlantic salmon stocks in three early life stages, and at four time points, to gain a better understanding of the genetic consequences of domestication. This was achieved by identifying genes and biological pathways differentially expressed between wild and domesticated stocks and, with the aid of their (reciprocal) hybrids, the heritability of the transcriptomic differences detected between stocks was investigated.

The meta-analysis presented in this chapter was undertaken to identify general trends that may apply to Atlantic salmon domestication regardless of the life stages. The same parameters, software and annotations were employed across experiments. In addition, multiple methods for functional analysis of the transcriptomic differences that occurred between the pure stock crosses were used in order to increase confidence in the results.

A number of pathways belonging to the *immune* and *nervous systems* and the *environmental information processing* biological function were down regulated in all early life stages of domesticated salmon, compared to their wild counterparts. The data also revealed a set of genes involved in growth and/or development that were up-regulated in the domesticated fish in multiple life stages. Additivity and maternal dominance were identified as the main form of inheritance of the transcriptomic differences detected between domesticated and wild strains. These findings are indicative of disruption to the natural allocation of resources, reduction of information acquisition and processing systems and loss of local adaptation of the domesticated fish, especially concerning immune function. The results suggest that the offspring of escapees may be heavily affected by the impact of domestication, and these alterations in gene expression are likely to be disadvantageous under natural conditions.

Chapter 3 - A comparison of gene transcription profiles of domesticated and wild Atlantic salmon (*Salmo salar L.*) at early life stages, reared under controlled conditions

3.1 Background

Commercial Atlantic salmon (*Salmo salar* L.) aquaculture was first initiated in Norway during the late 1960s, and has grown rapidly to become one of the most economically significant global aquaculture industries (FAO 2013). Current world-production is around 2 million tonnes, with Norway, Chile and Scotland representing the three largest producers. While this industry has been highly successful in terms of expanding production and reaching new consumer markets, this has not been achieved without increasing the potential for environmental impact. The question of environmental impacts following the escape of farmed salmon, and in particular the potential for genetic interactions with wild conspecifics, continue to provide key themes for scientific debate and public controversy (Ferguson et al., 2007; Hindar et al., 1991; Naylor et al., 2005).

Thousands of farmed salmon are reported to escape from aquaculture installations on a regular basis and, due to the probability of underreporting (Glover, 2010; Glover et al., 2008; Zhang et al., 2013), it has been estimated that the true number of escapees is likely to be significantly higher (Seagrov, H. and Urdal, 2006). Depending upon several factors such as fish age and time of escapement (Skilbrei, 2010a, 2010b), some farmed salmon manage to survive in the wild and enter freshwater where native salmon populations reproduce. Farmed escapees have been observed on the spawning grounds of native populations in Norway (Fiske et al., 2006; Gausen and Moen, 1991), the United Kingdom and Ireland (Walker et al., 2006; Webb et al., 1993; Youngson et al., 1997), Iceland (Gudjonsson, 1991) Western Canada (Volpe et al., 2014) and eastern North America (Morris et al., 2008). While the reproductive success

of farmed escapees is limited compared to wild salmon (Fleming et al., 2000, 1996), farmed salmon have been observed spawning in the wild (Seagrov, H. and Urdal, 2006; Webb et al., 1993; Webb, J H, Hay, D W, Cunningham, 1991), and genetic changes in native populations as a result of successful reproduction have been detected (Clifford et al., 1998; Crozier, 1993; Glover et al., 2012; Skaala et al., 2006).

A recent study of historical and contemporary samples from 20 Norwegian salmon rivers estimated cumulative introgression of farmed escaped salmon in native populations (Glover et al., 2013). Using a combination of single nucleotide polymorphisms (SNPs) and approximate Bayesian computation, these authors estimated introgression of farmed salmon reached nearly 50% in some rivers. This level of genetic introgression is of significant concern for two main reasons. First, wild Atlantic salmon populations are often genetically differentiated from one another and may be adapted to their specific rivers (Fraser et al., 2011; Garcia de Leaniz et al., 2007; McGinnity, P, Prodohl, P, Maoileidigh, N O, Hynes, R, Cotter, D, Baker, N, O'Hea, B and Ferguson, 2004; Taylor, 1991). Thus, invasion of a non-local fish may disrupt local adaptation. Second, farmed Atlantic salmon have been subject to selection for a range of traits since breeding programs were established in the early 1970´s (Gjedrem, 2010, 2000; Gjedrem et al., 1991). As a result, farmed salmon display a range of genetic differences to wild Atlantic salmon in a number of measured traits; for example, greatly increased growth rates under farming conditions (Glover et al., 2009; Solberg et al., 2013a, 2012; Thodesen et al., 1999), reduced predator awareness (Einum and Fleming, 1997), reduced genetic diversity in highly polymorphic genetic markers at the population level (Norris et al., 1999; Skaala et al., 2004), and altered gene-transcription patterns (Roberge et al., 2008, 2006). Furthermore, studies conducted in the wild have demonstrated that the offspring of farmed salmon display reduced survival compared to the offspring of wild salmon (Fleming et al., 2000; McGinnity et al., 2003, 1997; Skaala et al., 2012), an observation consistent with the

reported lower fitness of the offspring of hatchery fish in the wild (Araki and Schmid, 2010; Araki et al., 2008). Studies of the genetic differences between wild and domesticated salmon therefore represent an important contribution towards gaining understanding of the likely evolutionary consequences of interbreeding between escaped salmon and their wild conspecifics.

Forty years ago King and Wilson proposed that gene regulation governs evolution of anatomy, physiology and behaviour (Carroll, 2005; King and Wilson, 1975) and the development of broad-spectrum / high-throughput genomic approaches allows the theory to be tested. DNA microarrays, for example, are commonly used to simultaneously measure the mRNA expression levels of thousands of transcripts and have been available for salmonids since 2004 (Taggart et al., 2008; von Schalburg et al., 2005). As well as being employed to study genome-wide transcript expression, microarray experiments have been tailored to explore aspects of evolutionary processes, such as domestication in Atlantic salmon. In a series of microarray studies, Roberge and colleagues (Roberge et al., 2008, 2006) suggested that five to seven generations of selection for domestication may be sufficient to induce heritable alterations in transcription levels compared to wild populations. Of the differentially expressed genes that they detected, 16% displayed parallel changes among the strains, providing further evidence that artificial selection drives evolutionary changes at the gene transcription level (Roberge et al., 2006). Furthermore the authors suggested that, since most (82%) of the differentially expressed genes exhibited non-additive inheritance patterns, the consequences of introgression would likely to be difficult to predict (Roberge et al., 2008).

In the present study, microarray analysis was used to explore potential gene transcription / regulatory consequences of hybridisation between wild and domesticated salmon. In order to investigate genome wide transcript expression differences between wild and domesticated stocks, mRNA levels were compared for

yolk-sac and externally feeding fry originating from wild (Figgjo), domesticated (Mowi) and hybrid (Mowi ♀ x Figgjo ♂) populations reared under common conditions. Early life-history stages were focused upon, primarily to minimise transcriptional differences between the strains resulting solely from divergent inter-strain growth rates (up to three fold difference by four months post first feeding (Solberg et al., 2013a)). Furthermore, sampling during perceived periods of major physiological perturbation, *e.g.* hatching and swim up stages, were avoided, as individual variation during transition periods is likely to be critically influenced by sample timing. Body size differences in fish have been linked to developmental stage divergence and transcriptomic differences have been detected between size and age matched wild rainbow trout. Hence the exact methods employed to match life stages of wild and domesticated fish could influence the genes identified as differentially expressed between the stocks (White et al., 2013). With the aim of minimising the confounding factors described above, this study was designed to provide an insight into genetic differences and interactions between wild and domesticated salmon, since understanding such interactions is essential both for the support of sustainable aquaculture practices and for the maintenance of healthy wild stocks.

3.2 Methods

3.2.1 Biological samples

The farmed salmon juveniles used for the present study originated from the Norwegian Mowi strain maintained by Marine Harvest at Tveitevåg, Norway. This represents one of the oldest commercial salmon strains, and at the time of stripping, the eggs and sperm used to generate the family-groups originated from approximately the 10th generation. Established in the 1960s, the Mowi strain was initiated with wild Atlantic salmon from Southwestern Norway. Fish were primarily sourced from the River Bolstad and River Aaroy populations; characterised by large multi-sea winter fish and captured in the sea near the Oster and Sotra fjords. The Mowi strain was initially

selected for increased growth, late maturation and high flesh quality through phenotypic selection, however, a family-based breeding program which included expansion in the numbers of traits being selected for was initiated in 1999 (Glover et al., 2009). The Mowi strain has been demonstrated to display freshwater growth rates several times higher than various wild populations (Glover et al., 2009; K A Glover et al., 2006; Solberg et al., 2013a), and reduced survival compared to wild salmon under natural conditions when simultaneously planted out as eyed eggs (Skaala et al., 2012).

The wild salmon used in this study originated from the Figgjo River in south west Norway. This population represents one of the most abundant in Norway, and is characterised by small to medium-sized fish (typically 1-2 sea winter returns). In the period 15-17th October 2010, 24 wild fish were caught by rod and line angling in the river. These fish were transported to the local hatchery where they were held in tanks before being transported to the Matre research station in western Norway on 25th October 2010. These fish were confirmed to be wild based upon scale growth patterns (Lund and Hansel, 1991).

Both farmed and wild broodstock were stripped for gametes on 23rd November 2010. A total of 30 families were created; 10 of each of the following crosses: pure wild, Figgjo ♀ × Figgjo ♂; hybrid, Mowi ♀ × Figgjo ♂; pure domesticated, Mowi ♀ × Mowi ♂. Fertilised eggs were placed into single family incubators and were held under standard hatchery conditions. At the eyed egg stage on 22nd February 2011, families were pooled (30 eggs per family) into duplicate experimental groups, *i.e*. six tanks in total, and by 23rd March 2011 half of the eggs had hatched, these being termed 0°d post-hatch. The first sampling took place during fry yolk-sac re-absorption (256°d post-hatch) and then fish were transferred to heated (13^{o}C) first feeding tanks. Fry were fed on standard hatchery diet (Skretting) 24hr a day by automatic feeders according to a standard Skretting feeding table for appropriate temperatures. The second sampling took place 5 weeks into exogenous feeding (867°d post-hatch). The fish were starved

for 24hr prior to the second sample. For both sampling time points fry were euthanised with metacaine (Finquel® Vet, Scanvacc, Årnes, Norway) overdose, with yolk sac fry being placed into RNALater® (Life Technologies) and feeding fry being snap frozen on dry ice and stored at -70°C until homogenised.

The experiment was conducted in accordance with Norwegian regulations for the use of animals in research. No specific permits were required for this experiment because the fish were hatched and reared under standard aquaculture conditions without any form of experimental manipulation.

3.2.2 Microarray Experimental Design

Microarray interrogations were performed using a custom-designed, oligonucleotide microarray platform (Agilent) with 44 K probes per slide (Salar_2; Agilent Design ID:025520). This microarray has been described in detail elsewhere (Tacchi et al., 2011) and further used / validated in a number of subsequent studies (Martinez-Rubio et al., 2012; Morais et al., 2012; Tacchi et al., 2011). The design is logged with ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number A-MEXP-2065. Dual-label hybridisations were undertaken, with each experimental sample (Cy3 labelled) being competitively hybridised against a pooled reference control (Cy5 labelled) comprising equimolar amounts from each experimental RNA sample. The interrogations comprised 36 separate hybridisations; 3 states (wild x wild; farmed x wild, farmed x farmed) x 2 time-points (sac fry and fed fry) x 6 biological replicates.

	Sac fry	Feeding fry
Wild; F ♀ × F ♂	6 pools	6 pools
Hybrid; M ♀ × F ♂	6 pools	6 pools
Domesticated; M ♀ × M ♂	6 pools	6 pools

Table 3.1 A representation of the experimental design, which involved a total of 36 hybridisations, with each biological replicate comprising equal quantities of RNA from six individuals. A single array was excluded from the analysis as it failed quality filtering, hence only 5 pools of domesticated feeding fry were analysed

3.2.3 RNA Extraction and purification

Whole fry (n = 216) were homogenised rapidly in 8 × volume Tri Reagent (Sigma–Aldrich®, St. Louis, U.S.A.) using a Polytron mechanical homogeniser (Kinematica PT 1300 D, Lucerne, Switzerland) and the RNA extracted following the manufacturer's instructions. RNA quantity and quality were assessed by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.) and agarose gel electrophoresis respectively. For each hybridisation sample, equal amounts of total RNA from six individuals were pooled, column-purified (RNeasy Mini Kit, Qiagen, Crawley, UK), and then re-quantified and quality assessed as described above.

3.2.4 RNA amplification and labelling

Each pooled RNA sample was amplified (TargetAmp^{TM} 1-Round Aminoallyl-aRNA Amplification Kit, Epicentre Technologies Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. Following quality control (Nanodrop quantification and agarose gel electrophoresis) each aRNA sample was indirectly labelled and purified. Briefly, Cy dye suspensions (Cy3 and Cy5) in sufficient quantity for all labelling reactions were prepared by adding 40 µL high purity dimethyl sulphoxide (Stratagene, Hogehilweg, The Netherlands) per tube of Cy dye (PA23001 or PA25001; GE HealthCare, Little Chalfont, Bucks, UK). Each sample (2.5 µg aRNA) was denatured at 75°C for 5 min and then 3 µL 0.5 M $NaHCO_3$ pH8.5 and 1.5 µL Cy3 or 1.0 µL Cy5 dye was added achieving a total volume of 15 µL per reaction. Samples were incubated for an hour at 25^{o}C in the dark, purified using Illustra AutoSeq G-50 Dye Terminator Removal Kit (Qiagen GE Healthcare) and concentration, dye incorporation and purity were assessed via spectrophotometer (NanoDrop) with products also visualised on a fluorescent scanner (Typhoon Trio, GE Healthcare).

3.2.5 Microarray hybridisation and quality filtering

Hybridisation was performed over two consecutive days using the Agilent Gene Expression Hybridisation Kit (Agilent Technologies) as per manufacturer's instructions. For each reaction, 825ng Cy5 labelled reference pool and 825 ng Cy3 labelled individual samples were combined in 35 µL nuclease free water and then 20 µL fragmentation master mix added (consisting of 11 µL of 10X blocking agent, 2µL 25x fragmentation buffer and 7µL nuclease free water). The reactions were then incubated at 60^{o}C in the dark for 30 mins, chilled on ice, and mixed with 57 µL 2x GEx Hybridisation buffer (pre heated to 37^{o}C), Following centrifugation (18000 x g for 1 min) the samples were kept on ice until loaded (103 µL) in a semi randomised order onto the microarray slides. Samples from the six biological replicates were spread across different slides, Cy3 fluorescence content (dye incorporation rate x volume) was also taken into consideration. To aid scanning, samples with the most similar amounts of Cy3 were grouped on the same slide. Hybridisation was carried out in a rotating rack oven (Agilent Technologies) at 65^{o}C, 10 rpm over 17 hours.

Following hybridisation, slides were subject to a number of washing steps performed in Easy-Dip^{TM} slide staining containers (Canemco Inc., Quebec, Canada). First, each microarray and backing gasket was disassembled in Agilent Wash Buffer 1 and microarray slides were transferred to an Easy Dip rack submerged in Wash Buffer 1. Following 1 min incubation at room temperature (c. 20°C) and 150 rpm (Stuart Orbital Incubator), slides were briefly dipped into Wash Buffer 1 pre-heated to 31^{o}C, then placed into Wash Buffer 2 (31^{o}C) for 1 min at 150rpm. Finally, the slides were transferred to acetonitrile for 10 s and then Agilent Stabilization and Drying Solution for 30 s. The slides were then air dried in the dark and scanned within two hours.

Scanning was carried out at 5µm resolution on an Axon GenePix Pro scanner at 40% laser power. The "auto PMT" function was enabled to adjust PMT for each channel such that less than 0.1% of features were saturated and so that the mean intensity

ratio of Cy3:Cy5 signal was close to one. Agilent Feature Extraction Software (v 9.5) was used to identify features and extract background subtracted raw intensity values that were then transferred to GeneSpring GX (v.12) software where the quality filtering and normalisation steps took place. Intensity values ≤ 1 were adjusted to 1 and a Lowess normalisation undertaken. Stringent quality filtering ensured that features that represented technical controls, saturated probes, probe population outliers or probes which were not significantly different from the background were removed. Agilent feature extractions software was used to determine whether a probe was positive and significant based on a 2-sided t-test, indicating if the mean signal of a feature is greater than the corresponding background. A probe was retained if it was positive and significant in at least 75% of the arrays in any 2 of the experimental groups. This left 33,688 of the original 43,466 probes available for downstream analysis. A single array was excluded from the analysis as it was flagged as sub-standard by the feature extraction software and also appeared as a clear outlier on a Principal Component Analysis performed within Genespring in order to compare arrays. Thus 35 of the 36 arrays were statistically analysed.

Details of microarray experiment have been submitted to ArrayExpress under accession number E-MTAB-2578. The recording of the microarray experimental metadata complies with Minimum Information About a Microarray Experiment (MIAME) guidelines.

3.2.6 Microarray data analysis

Differentially expressed genes between the crosses were identified in GeneSpring using a number of statistical methods and criteria. For the entire data analysis, life stages were treated separately and to identify differentially expressed genes between experimental groups pairwise T-tests (unpaired unequal variance, $p \leq 0.01$) were performed and a minimum fold change of 1.3 applied. These lists formed the basis of the Venn diagram (Figure 3.1). In contrast, the functional analysis of the genetic

differences between wild and domesticated fish was based on less stringent criteria, with a p≤0.05 and with no fold change requirement and were further analysed in R v.3.0.2 (R Core Team, 2014). This enabled sufficient KEGG annotation for the pathway analysis which in turn narrowed the list of unique genes by further filtering on significant pathways using the gage function of the GAGE package (Generally Applicable Gene-set/Pathway Analysis)(Luo et al., 2009), q≤0.1) thereby increasing confidence despite the lenient initial comparison. The significant pathways (Table 3.2) were further analysed using the esset.grp and essGene functions (Luo et al., 2009) to identify non-redundant pathways and genes that changed over and above the noise level (Figures 2 and 3) respectively. Since pathways belonging to the human disease functional group are difficult to interpret in fish, this group was excluded from the gene enrichment analysis. Genes that were involved in any of the significantly perturbed pathways and changed beyond one standard deviation from the mean of all genes were subject to hierarchical clustering (Pearson correlation) and are presented on the heat maps using gplots package(Warnes et al., 2014). To look at heritability of differentially expressed genes between stocks, 1-way ANOVA (unequal variance) was performed with 10% FDR (Benjamini-Hochberg). To avoid repeat counting of the same gene, only transcripts that had BLASTx and/or KEGG annotation were chosen and where multiple probes were present for the same gene, the probe with the highest significance was chosen. For the unique genes obtained, additivity; α=(wild-domesticated)/2 and dominance parameters; δ=(wild+domesticated)/2-hybrid were calculated from normalised intensity values and α and δ/α were plotted using the ggplot2 package (Figure 3.4)(Wickham, 2009).

3.2.7 RT-qPCR validation

Expression of five selected genes was validated using real time quantitative polymerase chain reaction (RT-qPCR). Genes of interest were chosen based on their p-values in either of the life stages and/or fold changes across experimental groups.

Two additional ‘housekeeping’ genes were included in the analysis for normalisation purposes. Reference genes were selected from the literature (EF1A) or based on their constant/steady expression profile in the microarray analysis (MT28S). Details of the primer design and RT-qPCR are given in the Appendix.

cDNA was synthesised from 1µg of column-purified total RNA per sample using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), following manufacturer’s instructions, but using a mixture of the random primers (1.5 µL as supplied) and anchored oligo-dT (0.5 µL at 400 ng/µl). Negative controls lacking reverse transcriptase were included to check for genomic DNA contamination. A pool comprising similar amounts of all cDNA samples was used in a dilution series to determine primer efficiencies. The remaining cDNAs were then diluted 20-fold in water.

qPCR amplifications were carried out in duplicate 20 µL reaction volumes, containing either 5µL of cDNA (1/20 dilution) or no enzyme control (1/20 dilution) or serially-diluted cDNA pools (ranging from 1/10 to 1/640 dilution) or water (no template control) and 0.5µM each primer and 10µL ABgene Sybr Green (2x; Thermo Scientific, Wilmington, U.S.A.). All qPCR reactions were performed using the following thermal profile: initial activation at 95°C for 15 min, amplification through 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. Following the amplification phase, a melt curve analysis was performed to confirm the amplification of a single product. In addition, to determine the size and identity of the amplicons, agarose gel electrophoresis of amplicons was undertaken. Data were analysed in REST 2009 software (Pfaffl et al., 2002).

3.3 Results and Discussion

3.3.1 Differentially expressed transcripts between strains and life stages

For the purposes of statistical analysis, life stages were treated separately. In order to identify differentially expressed genes between experimental groups, pairwise T-tests

(unpaired unequal variance, $p \leq 0.01$, fold change ≥ 1.3) were used. The largest differences in transcription were observed between the domesticated and wild groups, however, it is interesting to note that there were fewer significantly differentially expressed transcripts between fish of hybrid and domesticated origin (176 in sac fry and 153 in feeding fry), than between wild and hybrids (300 and 567 respectively) (Figure 3.1A and 1B).

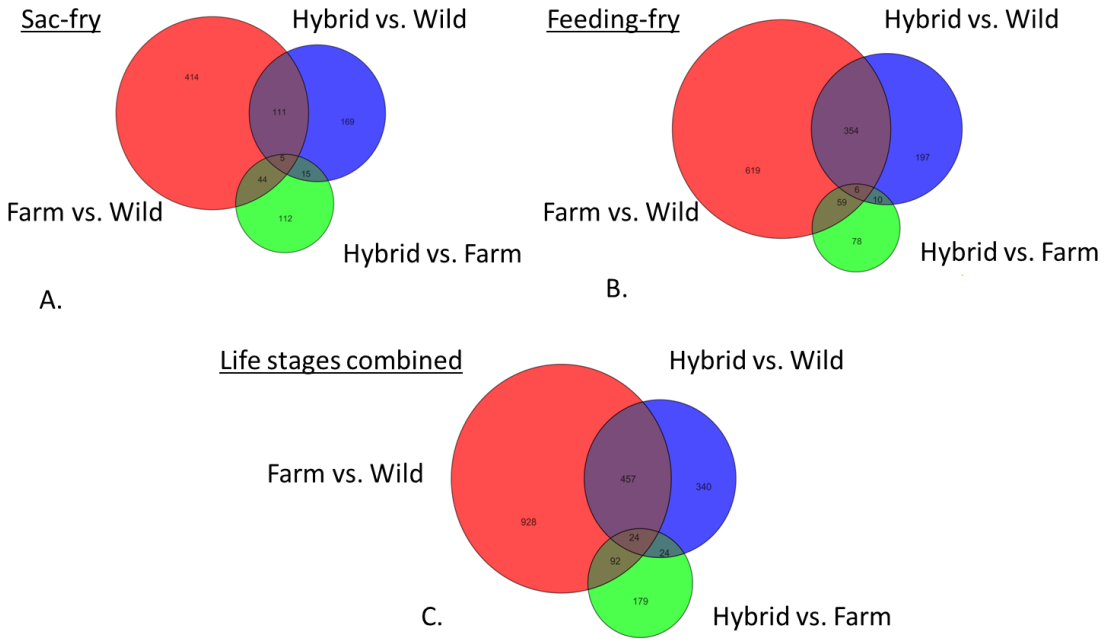


Figure 3.1 A comparison of the number of differentially expressed transcripts between groups and life stages, based on T-tests (unpaired unequal variance) without multiple testing correction, $p \leq 0.01$ and fold-change cut off at 1.3

Maternal effects might have contributed to the bias, as hybrid eggs were originated from domesticated females. In addition to direct genetic effects from the yolk sac, such as highly abundant maternal ribosomes and maternally deposited RNAs, other yolk sac components, such as hormones, proteins or nutrients can influence the offspring's genomic activity by modifying or interacting with its transcription factors or DNA structure (Bougas et al., 2013a). It was also noteworthy that there were over 1.8 times as many differentially expressed entities detected in the exogenous feeding stage than in the yolk-sac samples in the wild-domesticated and hybrid-wild comparisons (Figure

3.1). The initiation of exogenous feeding is known to alter gene expression through the activation of certain metabolic pathways, such as the glycolytic pathway enabling the utilisation of exogenous feeds or fatty acid pathways facilitating lipid metabolism and deposition (Mennigen et al., 2013). This was reflected in the observation that differentially expressed genes belonging to carbohydrate and lipid metabolism pathways were common in feeding fry, but not in sac fry. Furthermore, the hatchery diet employed, containing plant derivatives and thus poorly matching the usual diet of wild fish, might affect gene expression differentially in wild and domesticated stocks, and may thereby account for some of the differences detected in the feeding stage. However, the initiation of exogenous feeding did not increase the number of differentially expressed transcripts between domesticated fish and their hybrids, despite the expected fading of maternal effects in later life stages(Bougas et al., 2013a). Although some of the significantly differentially expressed genes overlapped between life stages, sampling at two time points revealed a number of life stage specific patterns (Figure 3.1C).

3.3.2 Functional classification of differentially expressed genes between wild and domesticated strains

It is difficult to make comparisons between studies at the level of differentially expressed genes due to the use of different stocks, life stages, tissues and microarray designs. Although common genes are rarely reported, biological pathways and even more so functional classes tend to overlap between studies (Roberge et al., 2006). To characterise the functional significance of the transcripts that were differentially expressed between wild and domesticated fish, we assigned KEGG annotations to them, unique genes were then subject to gene enrichment analysis (Table 3.2. and 3.3).

	KEGG functional group	*KEGG sub-group*	*KEGG pathway*	*Direction of perturbation*	*p.val*	*set.size*
Sac fry	Cellular Processes	Cell growth and death	**Oocyte meiosis**	Up regulated	**0.00212**	**15**
	Environmental Information Processing	Signal transduction	**Hippo signaling pathway**	Up regulated	**0.00128**	**15**
			Wnt signaling pathway		**0.00053**	**20**
	Genetic Information Processing	Folding, sorting and degradation	**Protein processing in endoplasmic reticulum**	Up regulated	**0.00186**	**36**
		Translation	**Aminoacyl-tRNA biosynthesis**	Up regulated	**0.00734**	**13**
			Ribosome		**0.00016**	**50**
			Ribosome biogenesis in eukaryotes		**0.00024**	**31**
			RNA transport		**0.00002**	**39**
	Cellular Processes	Transport and catabolism	**Phagosome**	Down regulated	**0.00042**	**37**
	Environmental Information Processing	Signal transduction	**NF-kappa B signaling pathway**	Down regulated	**0.00093**	**25**
		Signaling molecules & interaction	**Cytokine-cytokine receptor interaction**	Down regulated	**0.00000**	**26**
	Organismal Systems	Immune system	**B cell receptor signaling pathway**	Down regulated	**0.00133**	**16**
			Chemokine signaling pathway		**0.00000**	**38**
			Complement and coagulation cascades		**0.00385**	**21**
			Fc epsilon RI signaling pathway		0.00778	16
			Hematopoietic cell lineage		**0.00007**	**14**
		Nervous system	**Glutamatergic synapse**	Down regulated	**0.00152**	**19**
			Serotonergic synapse		**0.00303**	**16**
			Synaptic vesicle cycle		**0.00171**	**18**
	Metabolism	Lipid metabolism	**Glycerophospholipid metabolism**	Down regulated	**0.00781**	**10**
	Environmental Information Processing	Signal transduction	**NF-kappa B signaling pathway**	Two way perturbed	**0.00116**	**25**
			TNF signaling pathway		0.00368	19
		Signaling molecules and interaction	**Cytokine-cytokine receptor interaction**	Two way perturbed	**0.00001**	**26**
			Neuroactive ligand-receptor interaction		**0.00371**	**23**
	Organismal Systems	Development	**Osteoclast differentiation**	Two way perturbed	**0.00555**	**28**
		Immune system	**Chemokine signaling pathway**	Two way perturbed	**0.00343**	**38**
			NOD-like receptor signaling pathway		0.00416	14
			Toll-like receptor signaling pathway		**0.00240**	**19**
	Metabolism	Lipid metabolism	**Glycerophospholipid metabolism**	Two way perturbed	**0.00275**	**10**

Table 3.2 Significantly differentially represented KEGG pathways (multiple testing corrected p≤0.1) between wild and domesticated stocks in the sac fry stage, wild fish is considered as control. Set size is the number of genes included in the gene set test. Non-redundant pathways are shown in bold.

	KEGG functional group	*KEGG sub-group*	*KEGG Pathway*	*Direction of perturbation*	*p.val*	*set.size*
Feeding fry	Cellular Processes	Transport and catabolism	**Peroxisome**	Up regulated	**0.00014**	**27**
	Environmental Information Proc	Signaling molecules and interaction	**ECM-receptor interaction**	Up regulated	**0.01210**	**12**
	Organismal Systems	Circulatory system	Cardiac muscle contraction	Up regulated	0.00939	20
		Digestive system	**Fat digestion and absorption**	Up regulated	**0.00047**	**16**
			Pancreatic secretion		**0.00062**	**17**
			Protein digestion and absorption		**0.00004**	**23**
		Endocrine system	Adipocytokine signaling pathway	Up regulated	0.00435	15
			Insulin signaling pathway		**0.00000**	**19**
			PPAR signaling pathway		**0.00000**	**29**
	Metabolism	Amino acid metabolism	**Arginine and proline metabolism**	Up regulated	**0.00014**	**17**
			Glycine, serine and threonine metabolism		**0.00029**	**17**
		Carbohydrate metabolism	**Glycolysis / Gluconeogenesis**	Up regulated	**0.00001**	**25**
			Propanoate metabolism		**0.00084**	**12**
		Energy metabolism	Carbon fixation in photosynthetic organisms	Up regulated	0.00091	12
			Methane metabolism		**0.00048**	**12**
			Oxidative phosphorylation		**0.00058**	**59**
		Lipid metabolism	**Biosynthesis of unsaturated fatty acids**	Up regulated	**0.00004**	**12**
			Fatty acid degradation		0.00056	16
			Fatty acid elongation		0.00742	11
			Glycerolipid metabolism		0.00429	15
	Environmental Information Processing	Signal transduction	Jak-STAT signaling pathway	Down Regulated	0.00005	16
			NF-kappa B signaling pathway		**0.00000**	**28**
		Signaling molecules and interaction	**Cytokine-cytokine receptor interaction**	Down Regulated	**0.00000**	**38**
			Neuroactive ligand-receptor interaction		**0.00122**	**22**
	Organismal Systems	Immune system	**Antigen processing and presentation**	Down Regulated	**0.00267**	**22**
			Chemokine signaling pathway		0.00000	35
			Cytosolic DNA-sensing pathway		**0.00799**	**10**
			Fc epsilon RI signaling pathway		**0.00130**	**12**
			Fc gamma R-mediated phagocytosis		0.00407	17
			Toll-like receptor signaling pathway		0.00055	17
	Genetic Information Processing	Folding, sorting and degradation	**Proteasome**	Down Regulated	**0.00000**	**25**
	Environmental Information Proc	Signaling molecules and interaction	**Cytokine-cytokine receptor interaction**	Two way perturbed	**0.00000**	**38**

Table 3.3 Significantly differentially represented KEGG pathways (multiple testing corrected p≤0.1) between wild and domesticated stocks in the feeding fry stage, wild fish is considered as control. Set size is the number of genes included in the gene set test. Non-redundant pathways are shown in bold.

Transcriptional changes between wild and domesticated fish varied according to functional group life stage considered (Table 3.2 and 3.3). Among the differentially expressed transcripts, the ones relating to the immune system were significantly over-represented in both life stages. In addition, disproportionately large numbers of differentially expressed transcripts were detected for the nervous and digestive systems in sac fry and feeding fry respectively. An interesting parallel to this trend has been reported from transcriptomic comparisons between normal and dwarf lake white fish (*Coregonus* spp.), where the authors stressed the importance of survival functions in dwarf individuals and growth related functions in normal fish (Bernatchez et al., 2010). The majority of differentially expressed immune related transcripts were down-regulated in domesticated animals, whereas the opposite was observed for transcripts associated with the digestive system (Table 3.2 and 3.3.). Such apparent trade-offs between growth and immune response have also been documented in Atlantic salmon by previous authors (K. A. Glover et al., 2006). It has been suggested that selection for growth could therefore favour individuals with more active endocrine regulatory components (Fleming et al., 2002) and this is supported by the findings that most differentially expressed transcripts relating to the digestive system showed higher expression in domesticated individuals as did endocrine system related transcripts (Table 3.3.). In contrast, transcripts with nervous system and environmental information processing roles were mainly down-regulated in the domesticated strain, which might be explained by the relatively homogeneous and controlled environment experienced by domesticated individuals. Tymchuk and colleagues reported a down regulation of cell division in the brain of domestic rainbow trout, despite conducting their experiment on size-matched fish (Tymchuk et al., 2009). The relationship of wild : domesticated transcripts involved in energy metabolism, protein synthesis, stress and immune response, response to stimuli and digestion are in agreement between this study and previous studies investigating effects of domestication in salmonids (Debes et al., 2012; Roberge et al., 2008, 2006; White et al., 2013). Dishevelled Segment

Polarity Protein 2 (DVL2), a member of the Wnt signalling pathway, was hypothesised in previous work to show footprints of selection through domestication in Atlantic salmon (Martinez et al., 2013). Although oligo probes for this particular gene were not incorporated in the design of *Salar_2*, the Wnt signalling pathway was significantly up regulated in the sac fry stage.

A number of differentially expressed pathways were common between life stages, further increasing confidence in their significance. Toll-like receptor interaction, NF-kappa B signalling and cytokine-cytokine interactions pathways were down-regulated in the domesticated strain at both sampling points (Table 3.2. and 3.3). Toll-like receptors are primary sensors detecting a wide variety of microbial components and triggering innate immune responses through activating the transcription factor nuclear factor-kappaB, which controls the expression of inflammatory cytokine genes (Kawai and Akira, 2007). Cytokines have the ability to regulate endocrine activity and stress hormones and, in addition to immune activation they are likely to play a role in a number of interrelated processes, such as food intake efficiency, energy balance and tissue metabolism (Tort, 2011), and could thus provide a linking element between the differentially expressed pathways identified in this study.

To visualise expression patterns of the key genes belonging to identified significant pathways, hierarchical clustering was performed and expression intensities are shown on heat maps for the two life stages (Figure 3.2 and 3.3). Although universal transcript-level differences have not been identified when studying different wild and domesticated strains, there are a small number of genes that have been reported to be differentially expressed by more than one study. Parallel changes included ATP synthase, growth hormone receptor (Roberge et al., 2006), cytochrome (this study, (Debes et al., 2012; Roberge et al., 2006; White et al., 2013), solute carrier family members (this study (Debes et al., 2012; White et al., 2013),

Figure 3.2 Hierarchical clustering of the essential genes of the significant pathways for sac fry. Colour coding is based on normalised intensity values.

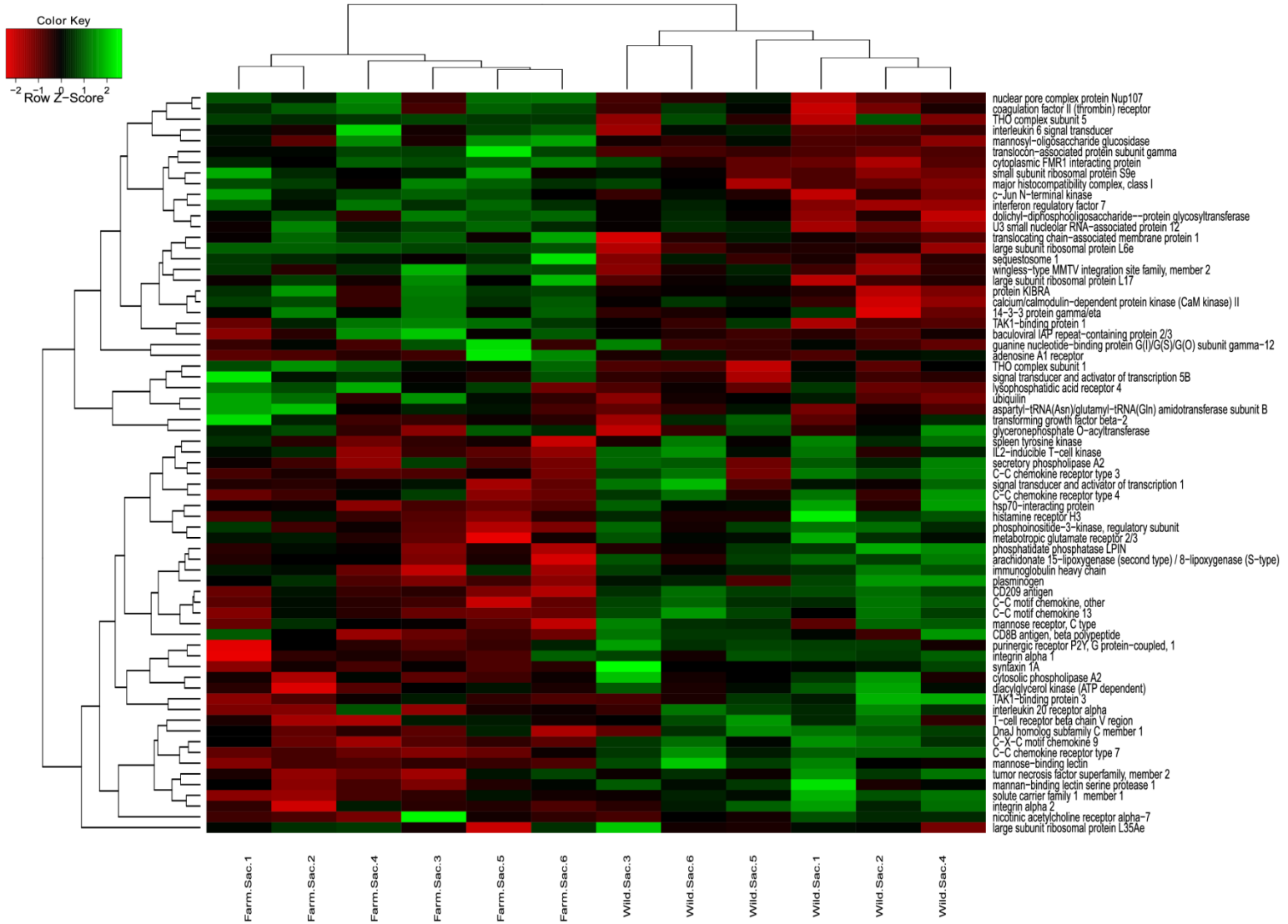
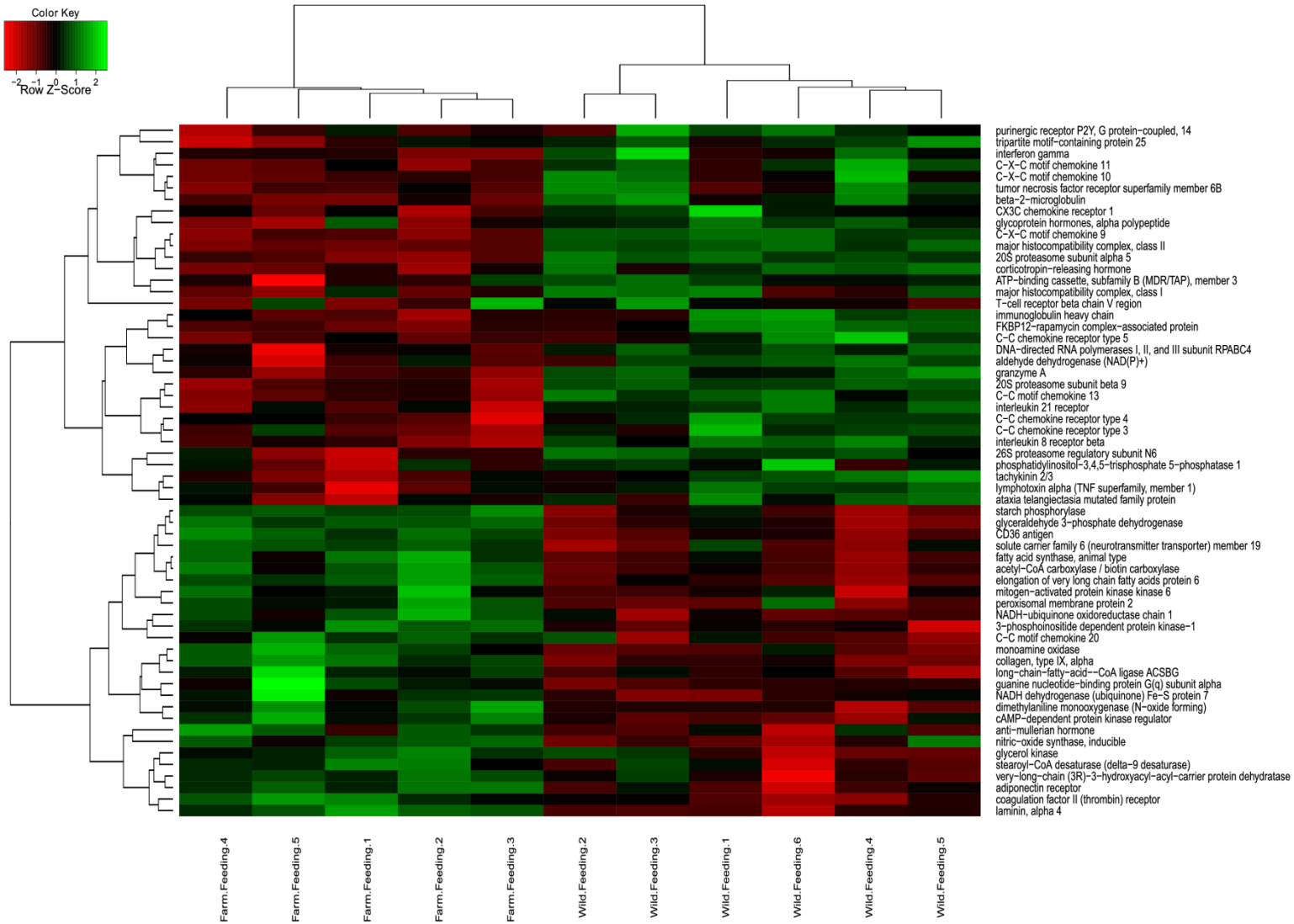


Figure 3.3 Hierarchical clustering of the essential genes of the significant pathways for feeding fry. Colour coding is based on normalised intensity values.



glyceraldehyde 3-phosphate dehydrogenase (this study, (Roberge et al., 2006) and malate/NADH dehydrogenase (this study, (Debes et al., 2012; Roberge et al., 2006). A number of immune related transcripts such as lectin and various CD and MHC family members were also reported by multiple sources, however their direction of change varies between studies (this study, (Debes et al., 2012; Roberge et al., 2006; Tymchuk et al., 2009; White et al., 2013).This might be due to the high specificity of the pathogen induced chemokine regulation (Alejo and Tafalla, 2011).

3.3.3 Heritability predictions of differentially expressed genes

To shed light on the inheritance patterns of the genes differentially expressed between stocks gene expression additivity was studied. 1-way ANOVAs were performed with multiple testing corrections (corrected $p \leq 0.1$) and only unique genes (see Materials and Methods for details); 25 in sac fry and 313 in feeding fry were included in the analysis. By calculating the ratio of the dominance parameter, δ=(wild+domesticated)/2-hybrid and the additive parameter, α=(wild-domesticated)/2 one can estimate the inheritance pattern of genes from their expression values. By definition a transcript whose hybrid gene expression value corresponds to the mid value of the parents is additive, whereas a transcript whose hybrid gene expression value resembles more closely one parent or another is dominant. $\delta/\alpha=0$ corresponds to a state of perfect within-locus additivity (i.e.; $\delta=0$) and $\delta/\alpha=1$ or -1 corresponds to complete dominance. According to logic and an assumption used by Renaut *et al*. (2009) in halving the intervals, we can presume that transcripts resemble:

-Additivity if $-0.5<\delta/\alpha<0.5$

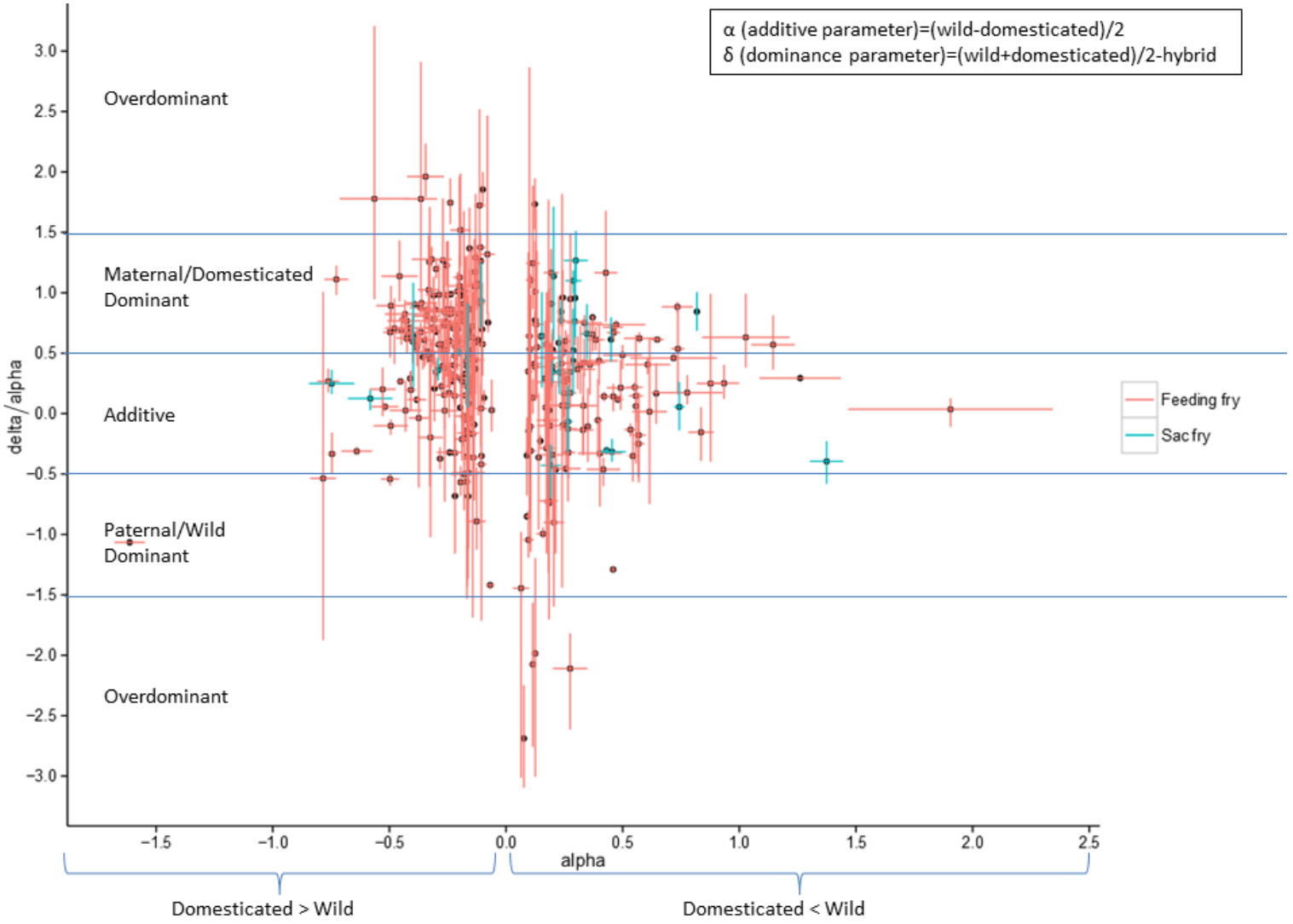
-Paternal/Wild dominance if $-1.5<\delta/\alpha<-0.5$

-Maternal/Domesticated dominance if $0.5<\delta/\alpha<1.5$

-Over-dominance if δ/α falls out of the interval -1.5-1.5

According to our results (Figure 3.4), most transcripts found to be differentially expressed between stocks showed either additive; 48% and 45% or maternal/domesticated dominant; 52% and 42.2% heritance patterns in sac fry and feeding fry respectively. In addition, 6.1% of transcripts were paternal dominant and 6.7% were over-dominant in the feeding stage. Among the over-dominant transcripts, the ones considered to be more similar to the mother's expression were approximately three times more abundant than the ones found to be closer to the father's. Additivity, as an important mode of inheritance between diverged intraspecific populations, has been reported in previous gene expression studies conducted on wild and domesticated salmon (Debes et al., 2012) and brook charr (Bougas et al., 2010) as well as on dwarf and normal lake white fish (Renaut et al., 2009). Additive genetic variation was also found to influence a number of traits in Atlantic salmon such as fitness, survival (Ferguson et al., 2007; Dylan J Fraser et al., 2010), growth and behaviour (Dylan J Fraser et al., 2010; Glover et al., 2009; Solberg et al., 2013a). In addition to additivity, the findings of this study are indicative of the relevance of a dominant inheritance pattern in wild-domesticated hybrids. However, since the hybrids in this study were produced only by crossing a domesticated dam with a wild sire, we are unable to conclude whether the dominance is purely caused by maternal effects or if the domesticated strain has a superior influence on the transcription of the offspring too. The importance of maternal dominance was highlighted by Bougas and colleagues when studying the transcriptional landscape of wild and domesticated brook charr hybrids. Similarly to the results reported here, their comparison of domesticated and anadromous wild fish revealed that 54.3% of the differentially expressed transcripts exhibited an additive inheritance pattern, 40% showed maternal, 5% paternal dominance, and a small number of transcripts were over/under dominant (Bougas et al., 2010).

Figure 3.4 Visual representation of heritability of annotated transcripts differentially expressed between experimental groups based on 1-way ANOVA (10% FDR). Error bars show the standard deviation between replicate arrays. Nine over-dominant, one dominant and one recessive transcript were excluded for ease of visualisation.



Contrary to the current findings, Debes *et al*. reported that 26.8% of the wild-domesticated Atlantic salmon hybrid transcripts showed wild dominance (Debes et al., 2012). There are a number of variables between the experiments that might account for the differences observed between the studies. First, since different tissue types (gill vs whole fry) were used in the studies, tissue specific gene expression might have affected the results. Second it is likely that the different parental strains crossed had different genetic architecture, which could have affected the gene expression of the offspring. In addition, Debes *et al*. report the use of reciprocal hybrids, whereas in this study, hybrid eggs originated only from domesticated animals. Third, since parental effects vary over time, and seem to be most pronounced at the yolk sac resorption stage, and tend to decrease over time, the sampling time-point selected could also have contributed to the gene expression differences of the hybrids (Bougas et al., 2013b). Indeed, in the current study a higher proportion of genes showed a dominant inheritance pattern at the yolk sac stage (52%) then during exogenous feeding (42%), suggesting stronger maternal influence at the earlier life stage. Tissue specificity, the time spent under selection pressure and the genetic architecture of the parental strains might have contributed to the disagreement between our results and a study reporting equal additive, recessive and dominant regulation when analysing the heritability of transcription in livers of wild and domesticated rainbow trout (White et al., 2013).

3.3.4 RT-qPCR validation of the results

Four significantly differentially regulated transcripts were chosen for further investigation via RT-qPCR, based on their p-values and fold changes. In addition, IGF-1 was also included in the RT-qPCR experiment due to its hypothesised functional importance in the process of domestication (Solberg et al., 2012) and despite the fact that no significant gene expression difference was detected for this transcript on the microarray. Although fold changes were generally low, a good correspondence of

expression ratio and direction of regulation was obtained between the microarray and RT-qPCR for most genes quantified (Table 3.4.).

	Sac fry				Feeding fry			
	Domesticated		Hybrid		Domesticated		Hybrid	
Target	**RT-qPCR**	**MA**	**RT-qPCR**	**MA**	**RT-qPCR**	**MA**	**RT-qPCR**	**MA**
MHCII	-1.48	-1.37	-1.17	-1.10	-1.95	-2.09	-1.24	-1.38
EPHX	1.27	2.24	1.20	1.57	1.23	2.08	1.20	1.55
IGF	-1.11	1.39	1.01	1.56	1.08	-1.14	1.05	1.79
Pesc	1.02	2.82	1.03	1.91	-1.15	2.43	-1.10	1.36
Poly10	-2.31	-6.72	-1.28	-1.78	-1.61	-3.19	-1.28	-1.63

Table 3.4 A comparison of gene expression ratios of domesticated and hybrid salmon with respect to wild individuals evaluated using RT-qPCR and microarray analysis. Microarray values are based on T-tests (unpaired unequal variance, p≤ 0.01 and FC>=1.3), whereas RT-qPCR ratios were obtained by REST2009 (p≤0.05). Non-significant values are highlighted in grey. Ratios lower than 1 are expressed as -1/ratio to obtain an equivalent value to ratios above 1.

Consistent with the microarray data, RT-qPCR results also showed no significant difference in expression of IGF-1 between experimental groups. In contrast, Solberg et al. found elevated IGF-1 mRNA levels in domesticated and hybrid Atlantic salmon head kidneys compared to those of wild fish (Solberg et al., 2012). The disagreement between our results might be due to the different strains, life stages and tissue types (head kidney vs whole fry) used in the studies.

3.4 Conclusions

This study investigated transcriptional differences between wild and domesticated Atlantic salmon at the early life-history stages, before developmental / growth rate between them could substantially influence experimental outcome. According to the results of this study, genetic information processing and translation pathways in particular are up regulated in domesticated fish whereas immune system related pathways are down regulated in the yolk sac stage. During early exogenous feeding , the digestive and endocrine systems as well as carbohydrate, energy and lipid metabolism pathways are more highly expressed in the domesticated strain, while

environmental information processing and immune pathways, especially those related to cytokines, are suppressed compared to those of wild stock.

While sampling complications following growth divergence between stocks need to be considered, it is important to study different life-stages to explore developmental state-specific differences between wild and domesticated individuals and the possible influence of common rearing on gene expression (*i.e*. translocation of wild fish into a hatchery environment). This study re-enforces the necessity of studying reciprocal hybrids in order to differentiate between maternal (and potentially epigenetic) and domestication effects influencing heritability. Finally, these data support the view that the effect of introgression is highly dependent on the population specific genetic architectures of the crosses (Normandeau et al., 2009; Roberge et al., 2008; White et al., 2013), thus studies conducted on multiple strains are essential to draw general conclusions regarding the outcome of genetic interactions between wild and farmed fish.

Chapter 4 - Comparing the transcriptome of embryos from domesticated and wild Atlantic salmon (*Salmo salar* L.) stocks and examining factors influencing heritability of expression

4.1 Background

The Atlantic salmon (*Salmo salar* L.) has been subject to domestication, including directional selection for economically important traits, since the aquaculture industry was first established in the early 1970s (Gjedrem, 2010, 1975). These breeding programs, which now extend beyond 10-12 generations, have been highly successful. For example, selection for growth rate, which has been the primary target of all Atlantic salmon breeding programs, has resulted in farmed fish attaining a body size 2-3 times greater than wild fish when reared under identical farming conditions (Glover et al., 2009; K. A. Glover et al., 2006; Solberg et al., 2013a, 2013b). However, economically important traits may not be beneficial in the wild, as demonstrated by reports of reduced survival of the offspring of farmed salmon *cf.* those of wild parents under natural conditions (Besnier et al., 2015; Fleming et al., 2000; McGinnity et al., 2003; Skaala et al., 2012). Given the magnitude of phenotypic and genotypic differences between wild and farmed salmon, it is feasible to investigate how domestication in general, as well as selection for specific traits, has altered both the structure and expression of the Atlantic salmon genome.

The early life-history of Atlantic salmon involves hatching from eggs planted in the gravel of rivers, absorption of the yolk-sac while resting in the gravel, emergence from the gravel in a process known as swim-up, and finally transition from endogenous to exogenous feeding. These critical, high mortality developmental stages play a major part in shaping the evolutionary trajectory of the individual and the population in

general (Einum and Fleming, 2000, 1999; Skaala et al., 2012). While numerous studies have investigated genetic differences between farmed and wild salmon, thus far relatively few have specifically targeted the critical early life stages. Exceptions include studies of fertilization success (Yeates et al., 2014), embryonic development speed and growth prior to exogenous feeding (Debes et al., 2013; Dylan J. Fraser et al., 2010; Solberg et al., 2014), mortality in the wild (Skaala et al., 2013), and gene transcription e.g.: (Bicskei et al., 2014; Roberge et al., 2008, 2006).

During the first phase of development, before the maternal-to-zygotic transition activates zygotic transcription, the embryo almost exclusively relies on maternal mRNAs and proteins (Tadros and Lipshitz, 2009) and until the initiation of exogenous feeding, pre- and post-eclosion embryos are largely dependent on maternally deposited yolk for energy provision (Kamler, 2007). Generally, eggs from farmed fish are reported to be inferior to wild eggs, due to nominally suboptimal maternal resources (Brooks et al., 1997). However, the extent of the differences observed varies across species and time and reduces with improving fish husbandry, feed formulation and rearing conditions (Bobe and Labbé, 2010). For example, the Atlantic cod aquaculture industry has yet to achieve optimal farming practices since fertilization and hatching of eggs from farmed broodstock are significantly lower than for wild broodstock (Lanes et al., 2012). In contrast, recent common garden studies have reported largely comparable fertilization success (*in-vitro*, (Lush et al., 2014) and hatching success (Solberg et al. 2014) between wild and domesticated Atlantic salmon stocks. The few differences detected were in egg size (affected indirectly through maternal body size) and hatching rate (Lush et al., 2014; Solberg et al., 2014), these two factors being considered to be interlinked and to differ between any two given populations (Mills, 1989). Although variability of these traits may be important to succeed under natural conditions (Einum and Fleming, 2000; Skaala et al., 2012),

these parameters do not, *per se,* serve to discriminate “high” and “low” quality eggs/embryos (Brooks et al., 1997).

Salmonid maternal effects have been well studied for easily measured phenotypic traits, such as egg and fry size, traits that have a significant impact on early survival (Einum and Fleming, 2000, 1999; Skaala et al., 2012). Studies at the transcriptional level are scarce. Debes *et al.* (2013) emphasized the fact that multi-generational genetics studies of salmonids rarely use reciprocal hybrids due to logistical constraints. Even when reciprocal hybrids are employed, data are often averaged across hybrids, hence obscuring maternal effects. A previous study exploring transcriptional differences in early life-history development between farmed and wild Atlantic salmon strains only included non-reciprocal hybrids that were generated by fertilizing domesticated eggs with wild milt (Bicskei et al., 2014). While this study documented dominant inheritance patterns exhibited by the F1 hybrids, the lack of fully reciprocal pedigrees precluded a further analysis of its primary source, *i.e.* domestication and/or maternal effects.

With the decreasing cost of broad-scale gene expression studies, transcriptomic profiling of fish embryos is starting to receive increased attention. Researchers have recently investigated how gene expression varies across embryonic development (Jantzen et al., 2011; Mommens et al., 2014; Škugor et al., 2014; Xu et al., 2011), have attempted to identify transcripts/markers associated with embryo quality (Lanes et al., 2013; Mommens et al., 2014), and have studied how gene expression in hybrid embryos is affected when divergent populations are crossed (Renaut et al., 2009). The present study employed a custom oligo-microarray as a tool to identify genes / gene pathways showing differential expression between embryos from wild and domesticated Atlantic salmon stocks reared under common conditions. By including reciprocal hybrids in the experimental design, heritability patterns were assessed to

specifically explore the relative importance of maternal *cf.* domestication effects in embryonic gene expression.

4.2 Methods

4.2.1 Biological samples

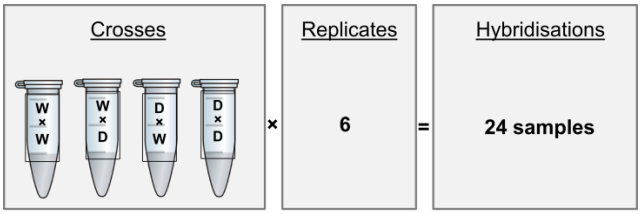
This study used experimental crosses involving the domesticated Norwegian Mowi strain, which has undergone approximately 10+ generations of directional selection for a range of economically important traits, and wild brood fish collected from the River Figgjo, located in the south west Norway. The characteristics of the Mowi strain have been investigated in a number of studies (Bicskei et al., 2014; Glover et al., 2009; Skaala et al., 2012; Solberg et al., 2015, 2013a, 2013b), and both strains have been previously described in detail elsewhere (Bicskei et al., 2014).

The experiment was initiated on 23rd November 2011 when gametes were stripped from four domesticated (MOWI) and four wild (Figgjo) salmon. Two independent sets of reciprocal crosses were established, each set using gametes from a pair of domesticated (D) and wild (W) parents to create four family combinations (i.e. pure wild, WxW; pure domesticated, DxD; and reciprocal hybrids W♀xD♂ and D♀xW♂). Fertilized eggs from each of the eight families were placed into individual family hatching trays under identical conditions. On 2nd February 2012, (approximately 410 °days post-fertilisation), eyed ova from each family (n = 30) were sampled. The eyed eggs were netted into an RNA stabilisation buffer (3.6 M ammonium sulphate, 18 mM Sodium Citrate, 15 mM EDTA, pH 5.2) and immediately pierced with a 25g syringe needle to aid rapid penetration of the preservative. Following overnight incubation at 8°C the RNAlater was drained and the eggs stored at −70°C until RNA extraction.

The experiment was conducted in accordance with Norwegian regulations for the use of animals in research. No specific permits were required for this experiment as the embryos were sampled prior to hatching.

4.2.2 RNA extraction and purification

Individual eyed eggs were homogenised in 1mL *Tri Reagent* (Sigma–Aldrich®, St. Louis, U.S.A.) using a *Mini-Beadbeater-24* (BioSpec Products Inc., Bartlesville, USA) and RNA was extracted following the manufacturer's instructions. RNA quantity and quality of individual embryos were assessed by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.) and agarose gel electrophoresis respectively. For each hybridisation sample (biological replicate), equal amounts of total RNA from eight individuals (4 per family x 2 families) were pooled per reciprocal cross type (WW, DD, DW or WD) and then re-quantified and quality assessed as described above (Figure 4.1).



Each sample is a pool of 8 individuals (4 from 2 families), hence a total of 192 individuals are being studied.

Figure 4.1 A schematic representation of the experimental design.

4.2.3 Microarray experimental design

Microarray analysis was performed using a custom oligonucleotide microarray platform (Agilent) with 4 x 44 K probes per slide (Salar3; ArrayExpress Accession number A-MEXP-2400). The general design of the microarray has been described in detail elsewhere (Tacchi et al., 2011) and further used/validated in a number of subsequent studies e.g.: (Bicskei et al., 2014; De Santis et al., 2015a; Martinez-Rubio et al., 2012; Morais et al., 2012; Tacchi et al., 2011).

Dual-label hybridisations were undertaken, with each experimental sample (Cy3 labelled) being competitively hybridised against a pooled reference control (Cy5 labelled) that comprised equimolar amounts from each experimental RNA sample. Thus every experimental sample was assessed relative to a single common sample, allowing a full range of inter-state comparisons. The interrogations involved 24

separate hybridisations; 4 reciprocal cross types (W♀ x W♂, D♀ x W♂; W♀ x D♂, D♀ x D♂) x 6 biological replicates (each replicate comprising RNA from 8 different individuals; 4 each from 2 families) (Figure 4.1).

4.2.4 RNA amplification and labelling

RNA from each biological replicate (pool of 8 individuals) was amplified (TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit, Epicentre Technologies Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. Following quality control (Nanodrop quantification and agarose gel electrophoresis) aRNA samples were indirectly fluorescently labelled and purified. Briefly, dye suspensions (Cy3 and Cy5) in sufficient quantity for all labelling reactions were prepared by adding 42 µL high purity dimethyl sulphoxide (Stratagene, Hogehilweg, The Netherlands) per tube of Cy dye (PA23001 or PA25001; GE HealthCare, Little Chalfont, Bucks, UK). Individual amplified samples (2.5 µL aRNA in 10.5 µL H_2O) were denatured at 75°C for 5 min and then 3 µL 0.5 M $NaHCO_3$ pH8.5 and 1.5 µL Cy3 dye added.. The common reference pool was similarly labelled, but prepared in a single large scale reaction; i.e. 50 µg pooled aRNA in 210 µL H_2O, heat denatured, with 60 µL 0.5 M $NaHCO_3$ pH8.5 and 20 µL Cy5 dye then added. All samples were incubated for an hour at 25^{o}C in the dark, and purified through Illustra AutoSeq G-50 Dye Terminator columns (Qiagen GE Healthcare). Dye incorporation and purity of all reactions were assessed spectrophotometrically (NanoDrop), with products also visualised on a fluorescent scanner (Typhoon Trio, GE Healthcare).

4.2.5 Microarray hybridisation and quality filtering

All hybridisations were performed at the same time using the Agilent Gene Expression Hybridisation Reagent Kit (Agilent Technologies) as per manufacturer's instructions. For each reaction, 825ng of Cy5 labelled reference pool and 825 ng of a Cy3 labelled test sample were combined in 35 µL water and then 20 µL fragmentation master mix added (11 µL of 10x blocking agent, 2µL 25x fragmentation buffer and 7µL water). The

reactions were then incubated at 60^{o}C in the dark for 30 mins, chilled on ice, and mixed with 55 µL 2x GEx Hybridisation buffer (pre heated to 37^{o}C). Following centrifugation (18000 x g for 1 min) the samples were kept on ice until loaded (103 µL) onto the microarray slides (four arrays per slide). Samples from the six biological replicates were divided across different slides. Hybridisation was carried out in a rotating rack oven (Agilent Technologies) at 65^{o}C, 10 rpm over 17 hours.

Following hybridisation, the microarray slides were washed in Easy-DipTM slide staining containers (Canemco Inc., Quebec, Canada). First, a 1 min incubation at room temperature (c. 20°C) in Wash Buffer 1 was performed, with gentle shaking at 150 rpm (Stuart Orbital Incubator). Slides were briefly dipped into Wash Buffer 1 pre-heated to 31^{o}C, then placed into Wash Buffer 2 (31^{o}C) for 1 min at 150 rpm. Finally, the slides were transferred to acetonitrile for 10secs and finally into Agilent Stabilization and Drying Solution for 30 secs. The slides were then air dried and scanned within 3 hrs.

Slides were scanned at 5 µm resolution on an *Axon GenePix Pro* scanner at 70% laser power. The “auto PMT” function was enabled to adjust PMT for each channels such that less than 0.05% of features were saturated and the mean intensity ratio of Cy3:Cy5 signal was close to one. *Agilent Feature Extraction Software (v 9.5)* was used to identify features and extract background subtracted raw intensity values that were then transferred to *GeneSpring GX (version 13)* software (Chu et al., 2001), where the quality filtering and normalisation steps were applied. Intensity values ≤ 1 were adjusted to 1 and a Lowess normalisation undertaken. Stringent quality filtering ensured that features that represented technical controls, saturated probes, probe population outliers or probes which were not significantly different from the background (based on a two sided t-test implemented in the Feature Extraction software) were removed. Finally probes were retained if they were positive and significant in at least 75% of the arrays in any two of the experimental groups. As a result 31491 probes passed quality control and were further analysed.

Details of the microarray experiment have been submitted to *ArrayExpress* under accession number E-MTAB-3677. The recording of the microarray experimental metadata complies with the Minimum Information About a Microarray Experiment (MIAME) guidelines.

4.2.6 Microarray data analysis

Statistical analysis (T-test and ANOVA) was performed in *GeneSpring software (version 13)*, whereas *R software* (R Development Core Team, 2008) was used for functional analysis (GAGE) and graphing. Details of each analysis are provided below. To minimize repeat counting the same gene, only transcripts that had BLAST (Altschul et al., 1990) and/or KEGG annotation (Moriya et al., 2007) were considered in downstream analysis, and where multiple probes were present for the same gene, the probe with the lowest *p*-value was chosen.

Functional analysis of the genetic differences between offspring of wild or domesticated pure stocks was performed via the *gage* function of the *GAGE package* (Generally Applicable Gene-set/Pathway Analysis) (Luo et al., 2009). Gene set tests establish correlations between functional groups and phenotype by detecting small but coordinated changes in gene expression (Luo et al., 2009).The analysis employed '1ongroup' comparison (pairwise comparisons between domesticated fish replicates vs the average of wild fish) and, as generally applied, results were considered significant if the corrected *p*-value was ≤ 0.1. For ease of visualization and a more focused interpretation, pathways that were perturbed in both directions (2d) *i.e.* transcripts that were not restricted in terms of their direction of change, were further filtered by applying a *p*-value cut off of 0.02. For a default ($p \leq 0.1$) 2d pathway list, see Appendix Table 4.2. Since pathways belonging to the human disease functional group are particularly difficult to interpret in fish, this group was excluded from the gene enrichment analysis. Significant pathways were further explored using the *essGene* function (Luo et al., 2009) to identify key genes. The package *ggplot2* (Wickham,

2009) was employed to graphically represent transcripts in significantly perturbed pathways that varied > 1SD from the mean of all transcripts and were significantly different between domesticated and wild strains (T-test unpaired unequal variance, $p \leq 0.05$). Where transcripts were represented in multiple KEGG groups, the function that had the most gene associations from the overall list was assigned to them.

To identify differentially expressed transcripts between embryos of domesticated and wild origin, a T-test (unpaired unequal variance, Benjamini-Hochberg multiple testing correction, corrected $p \leq 0.05$) was performed and a fold change filter ≥ 1.25 applied. Following hierarchical clustering (Pearson correlation), expression profiles of unique differentially expressed transcripts across stocks were visualized as heatmaps (*gplots* package; (Warnes et al., 2014).

To explore heritability of differentially expressed genes between stocks, one-way ANOVA (unequal variance) was performed with 10% FDR (Benjamini-Hochberg) and Student Newman-Keuls (SNK) post-hoc analysis. Differentially expressed transcripts were assigned to the following heritability categories:

i) Maternal effect: differentially expressed between
W♀ x W♂ vs D♀ x W♂ or D♀ x D♂ vs W♀ x D♂
ii) Paternal effect: differentially expressed between
W♀ x W♂ vs W♀ x D♂ or D♀ x D♂ vs D♀ x W♂
iii) Parental effect: influenced by both maternal and paternal effects
iv) Maternal only: unique to maternal effect
v) Paternal only: unique to paternal effect

For normalised intensity values (*ni*) of unique differentially expressed genes obtained: α = additivity = $(W_{ni} - D_{ni})/2$ and δ = dominance = $((W_{ni} + D_{ni})/2) - hybrid_{ni}$ were calculated. The values for α and δ/α were plotted using the *ggplot2* package (Wickham, 2009). A transcript whose expression value in hybrids is midway between that of the parents is additive (perfect additivity: $\delta/\alpha = 0$). A transcript whose hybrid

gene expression value resembles one of the two parents more closely is dominant (domesticated dominance, δ/α = 1; wild dominance, δ/α = -1). Group memberships were assigned as follows by halving the intervals:

- additivity, if $-0.5 < \delta/\alpha < 0.5$
- wild dominance, if $-1.5 < \delta/\alpha < -0.5$
- domesticated dominance, if $0.5 < \delta/\alpha < 1.5$
- over-dominance, if δ/α falls outside the interval −1.5-1.5.

For ease of plot interpretation, genes with $|\delta/\alpha| > 5$ were excluded from the scatter graph but were considered in the heritability table.

4.3 Results

4.3.1 Functional analysis

For the functional analysis, KEGG annotation was used. Approximately 62% of the probes that passed quality filtering had KO numbers assigned and about 50% of these returned unique annotations. Hence a total of 6037 genes were included in the gene set enrichment analysis, revealing a range of pathways displaying significant differential gene expression between the embryos of wild and domesticated parentage (Table 4. 1). The *ECM-receptor interactions* pathway was identified as down-regulated in domesticated fish, whereas pathways involved in *genetic information processing* and *metabolism* functions were up-regulated. Pathways involved in *genetic information processing* had a role in *mRNA translation*, whereas *metabolism* pathways comprised those associated with *carbohydrate*, *lipid* and *energy metabolism*. In addition, the most significant two-way perturbed pathways were related to *environmental information processing*; *cell signaling*, in particular, and *organismal systems*; including *digestive*, *immune* and *nervous systems*. Most differentially expressed transcripts and major contributors to these significant pathways were members of signal transduction pathways (Figure 4.2). Further KEGG functional groups displaying more than 10

differentially expressed genes included the *immune system*, *cell communication* and *signaling molecules and interaction.* There was considerable gene overlap between these groups. Data is not shown.

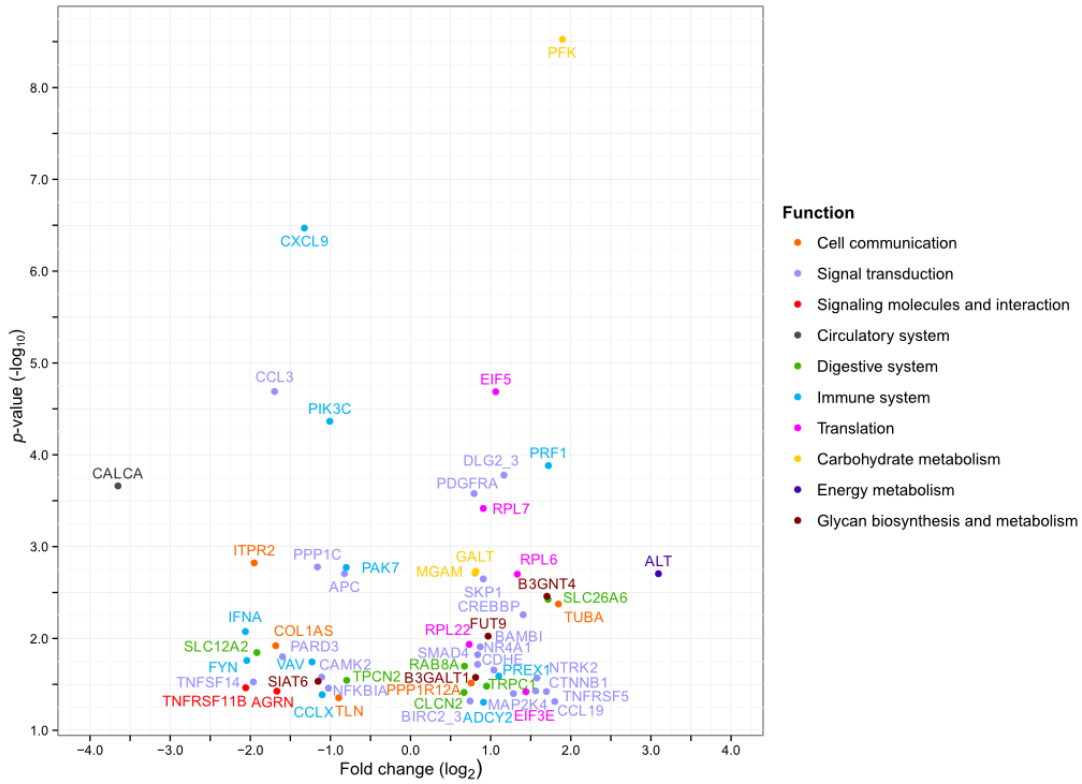


Figure 4.2 Key genes of the perturbed pathways. Differentially expressed genes (T-test $p \leq 0.05$) between wild and domesticated embryos and identified as essential for the pathways perturbed between pure stocks (Table 4. 1). Genes are plotted according to $\log_2$ fold change (domesticated vs wild) and $-\log_{10}$ *p*-value (T-test), and color-coded by biological function.

4.3.2 Expression profiling

T-tests identified 165 transcripts showing significantly different expression between embryos of domesticated and wild parentage, corresponding to 123 unique annotated transcripts. Hierarchical clustering of the differences revealed both additive and dominant behaviours (Figure 4.3 and 4.4). The most pronounced clusters were those indicative of maternal influence, such as the bottom cluster of Figure 4.3 and the top cluster of Figure 4.4. Both clusters contained several *cytochrome*-related genes.

Table 4.1 Differentially expressed pathways in domesticated vs wild embryos. KEGG based functional representation of the pathways differentially perturbed between wild and domesticated embryos and their significance in a previous study conducted on sac and feeding fry.

KEGG functional group	KEGG sub-group	KEGG Pathway	*p*-value	Direction of perturbation	Sac fry Bicskei *et al.* 2014	Feeding fry Bicskei *et al.* 2014
Cellular Processes	Cell communication	Focal adhesion	0.00051	Two way perturbed		
		Gap junction	0.00036			
Environmental Information Processing	Signal transduction	Hippo signaling pathway	0.00040		Up-regulated	
		MAPK signaling pathway	0.00101			
		NF-kappa B signaling pathway	0.00021		Down-regulated	
		Wnt signaling pathway	0.00213		Up-regulated	
	Signaling molecules and interaction	Cell adhesion molecules (CAMs)	0.00069/0.00144	Up/Two way		
		Cytokine-cytokine receptor interaction	<0.00001	Two way perturbed	Down-regulated	
		Neuroactive ligand-receptor interaction	0.00001		Two way perturbed	Down-regulated
		ECM-receptor interaction	0.00016	Down		Up-regulated
Organismal Systems	Circulatory system	Vascular smooth muscle contraction	0.00032	Two way perturbed		
	Development	Osteoclast differentiation	0.00019		Two way perturbed	
	Digestive system	Mineral absorption	0.00011	Up		
		Pancreatic secretion	0.00164	Two way perturbed		Up-regulated
		Salivary secretion	0.00117			
	Endocrine system	GnRH signaling pathway	0.00014			
	Immune system	Chemokine signaling pathway	0.00017		Down-regulated	
		Fc epsilon RI signaling	0.00026			
		Natural killer cell mediated cytotoxicity	0.00004			
		T cell receptor signaling pathway	0.00002			
	Nervous system	Glutamatergic synapse	0.00154		Down-regulated	
		Long-term potentiation	0.00001			
Genetic Information Processing	Translation	Ribosome	0.00383	Up	Up-regulated	
		RNA transport	0.00174			
Metabolism	Carbohydrate metabolism	Fructose and mannose metabolism	0.00183			
		Galactose metabolism	0.00168			
	Energy metabolism	Carbon fixation in photosynthetic organisms	0.00494			Up-regulated
	Glycan biosynthesis & metabolism	Glycosphingolipid biosynthesis - lacto & neolacto series	0.00316			
	Lipid metabolism	Sphingolipid metabolism	0.00229			

Figure 4.3 Up-regulated differentially expressed transcripts. Hierarchical clustering of the expression profiles of unique transcripts up-regulated in domesticated embryos compared to wild embryos.

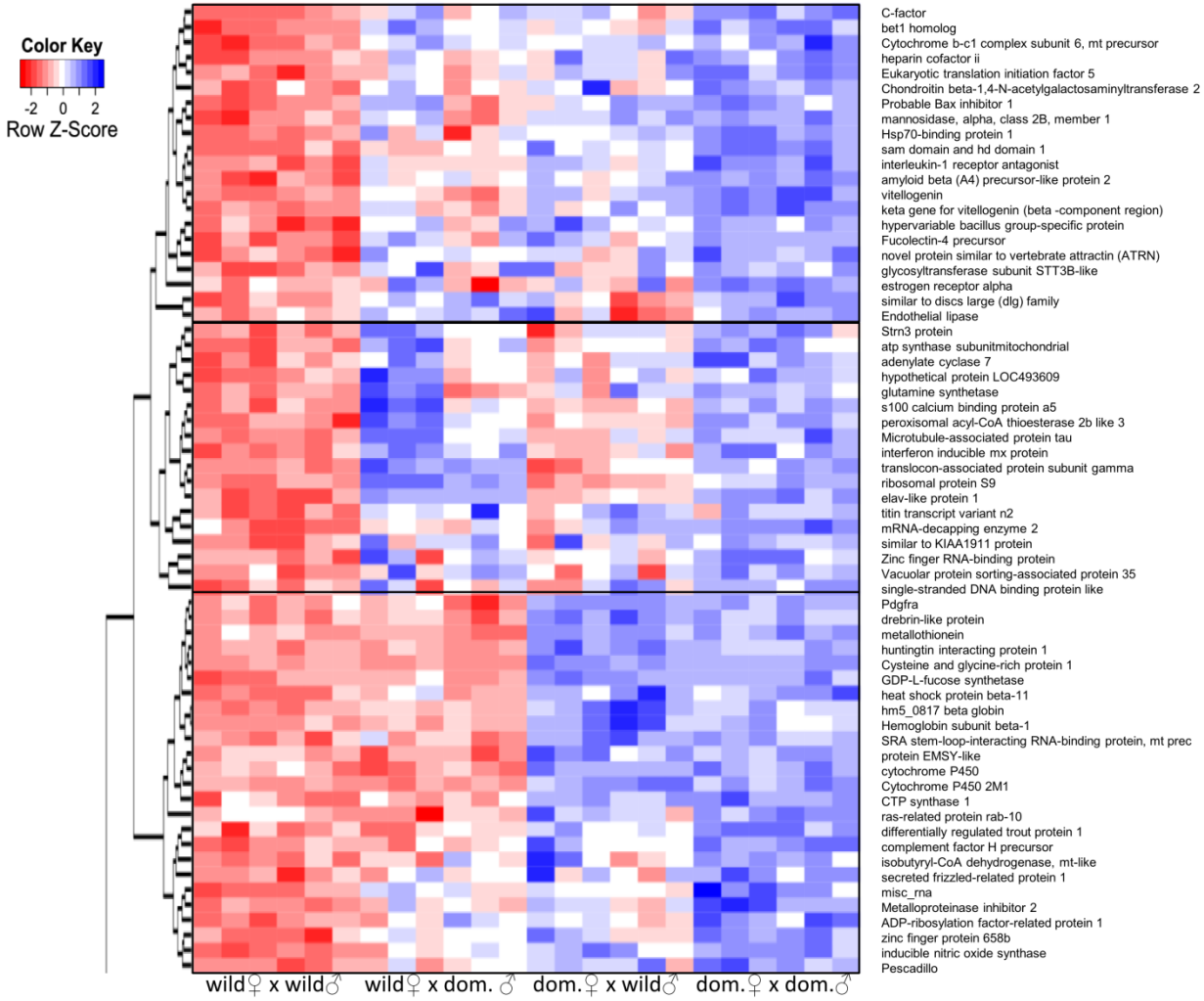
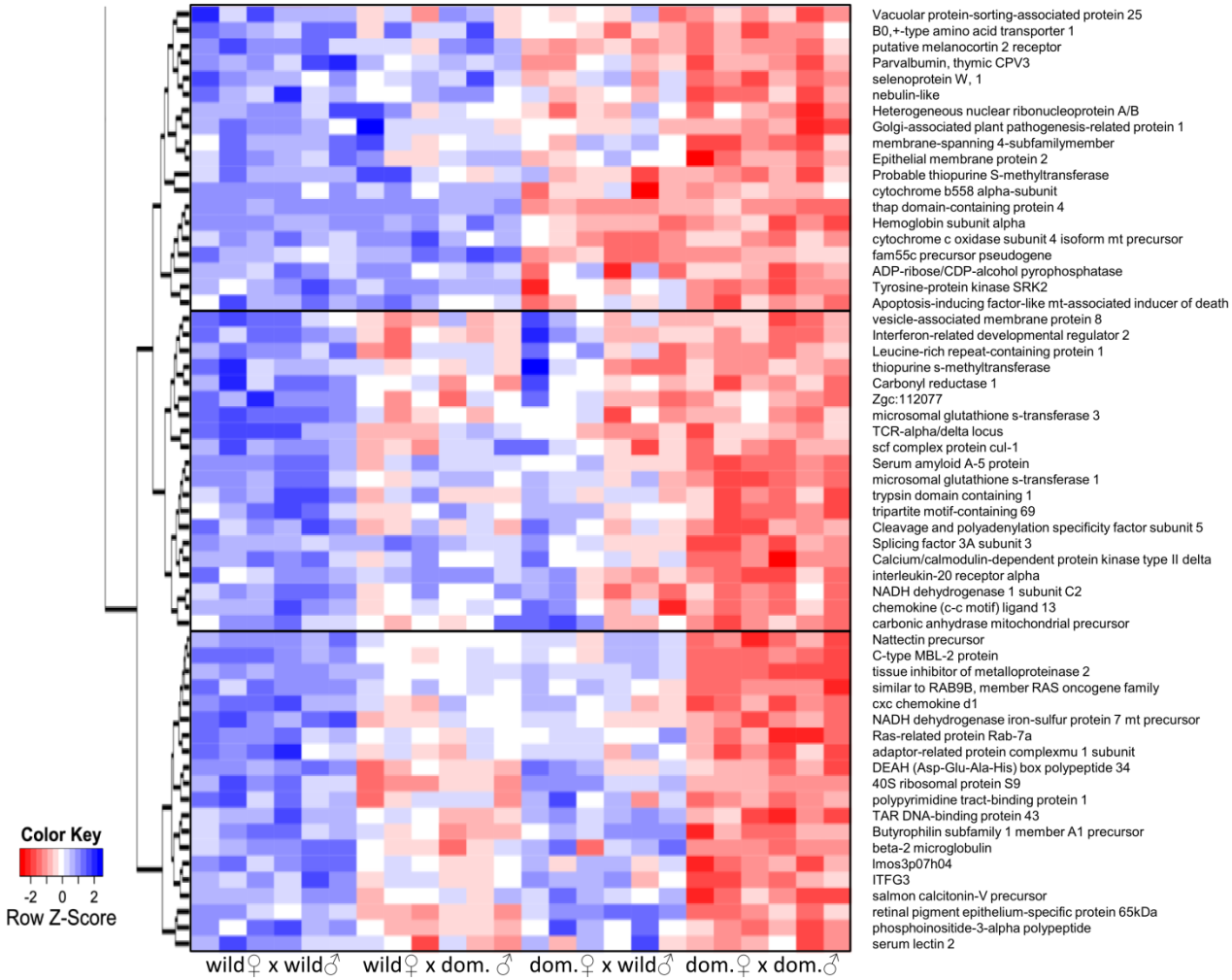


Figure 4.4 Down-regulated differentially expressed transcripts. Hierarchical clustering of the expression profiles of unique transcripts down-regulated in domesticated embryos compared to wild embryos.



4.3.3 Heritability analyses

To further investigate the significance of the parental effects indicated by expression profiling, additive and dominance parameters were calculated and plotted (Table 4.2 and Figure 4.5). Out of 208 transcripts showing differential expression among the four experimental groups by one-way ANOVA, only two were found to be significantly different between the pure crosses and were not considered further. There were no observed differences between hybrid × hybrid crosses that were not also seen between hybrid × pure crosses (Figure 4.6).

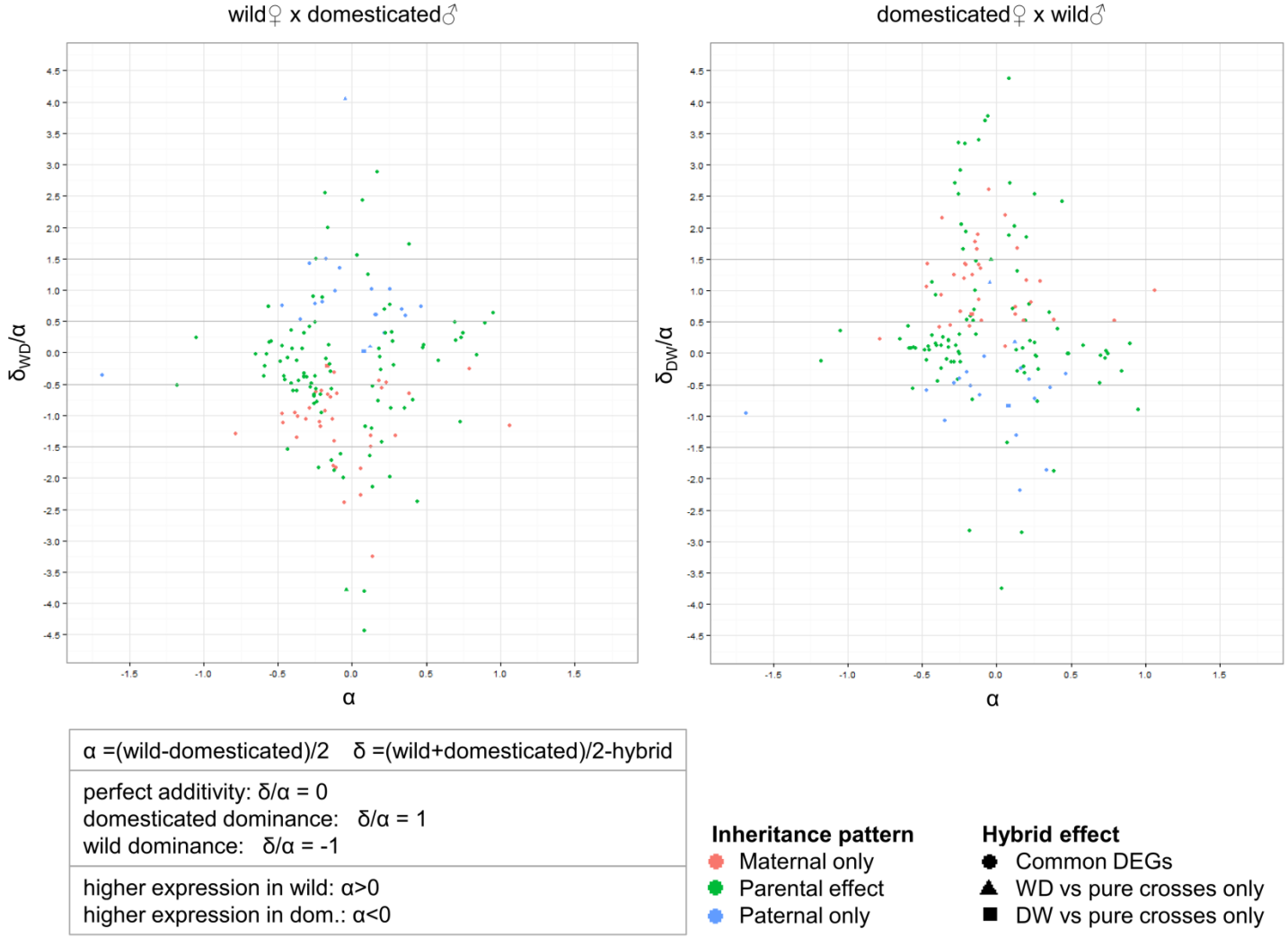
The remaining 206 differentially expressed transcripts identified, corresponded to 165 unique genes that were further analysed. The vast majority of the differences (153 genes) were shared by both hybrid crosses, whereas an additional nine and three genes were unique to either W♀ × D♂ or D♀ × W♂ hybrids respectively. For reciprocal hybrids, most transcripts exhibited either intermediate expression (33.3% and 42.3%) or dominance/over-dominance (27.8%/18.5% and 23.1%/21.2%, in the reciprocal hybrids respectively) favouring the maternal strain (Table 4. 2). However, W♀ × D♂ hybrids showed a stronger combined (wild or domesticated dominance) dominance effect (42% *vs.* 32.1%) and weaker additive effect (33.3% *vs*. 42.3%) compared to D♀ × W♂ hybrids.

Table 4.2 Proportions of the differentially expressed genes displaying various inheritance patterns. Based on a heritability analysis of the differentially expressed genes and a comparison of the inheritance patterns to a previous study conducted in sac and feeding fry. For explanation of the various categories see the materials and methods section.

Life stage	Hybrid type	Unique genes	Wild over-dominant	Wild dominant	Additive	Domesticated dominant	Domesticated over-dominant	Experiment
Embryo	W♀ × D♂	162	18.5%	27.8%	33.3%	14.2%	6.2%	Current study
	D♀ × W♂	156	4.5%	9.0%	42.3%	23.1%	21.2%	
Sac fry		25	0.0%	0.0%	48.0%	52%	0.0%	Bicskei *et al.* 2014
Feeding fry		313	1.6%	6.1%	45.0%	42.2%	5.1%	

Since most of the transcriptomic differences detected between strains were shared by both reciprocal hybrids, their expression could be compared to that of the pure crosses to determine whether these were primarily influenced by domestication or parental factors. Visualisation of the dominance behaviour (Figure 4.5) showed that most transcripts differentially expressed between stocks were either additive or maternally dominant. For ease of visualization, 15 over-dominant genes were excluded from the scatterplot (Figure 4.5), due to large $|\delta/\alpha|$.

Figure 4.5 Heritability predictions of the differentially expressed genes between stocks for the two hybrid stocks. DEG: differentially expressed gene, WD: wild♀ × domesticated♂, DW: domesticated♀ × wild♂



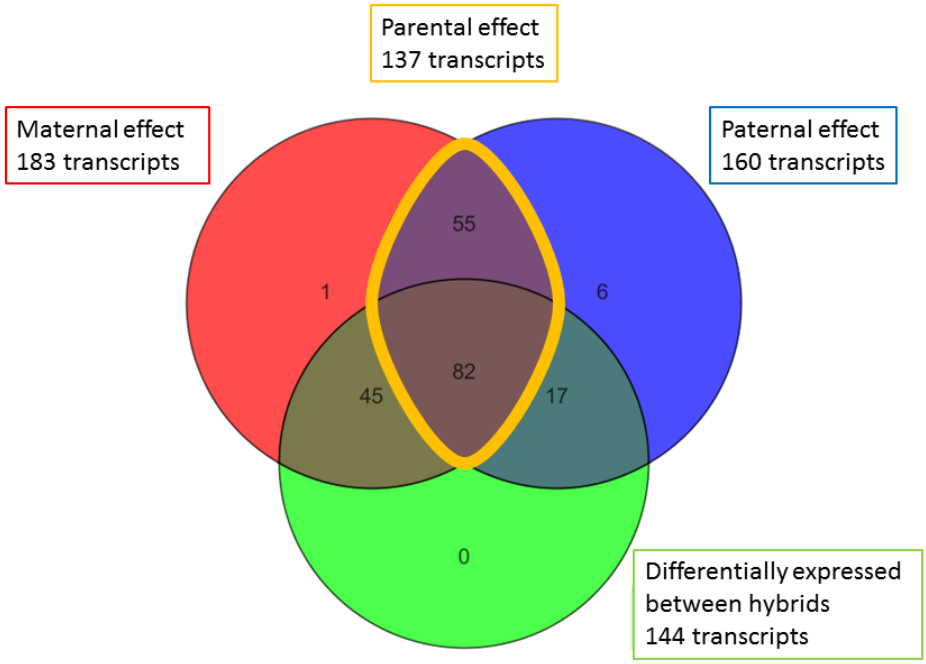


Figure 4.6 The number of transcripts differentially expressed between stocks and their inheritance pattern. Differences observed between hybrid and pure crosses are categorized as influenced by maternal, paternal or parental effects (see Methods for details). The number of differentially expressed transcripts identified between hybrid crosses is also shown.

4.4 Discussion

The first microarray studies comparing genome wide gene transcription of Atlantic salmon fry reported that 5-7 generations of domestication selection had induced heritable changes of gene expression in cultured relative to wild counterparts (Roberge et al., 2008, 2006). Observed differences occurred in common pathways but did not necessarily involve identical genes within a given pathway, this being ascribed to differences in 'genetic architecture' between stocks. A more recent study (Bicskei et al., 2014) demonstrated that whilst common differences could be observed between life stages, a number of the key pathways affected were stage-dependent. Since the experimental designs of these earlier studies included analysis of D♀ × W♂ hybrids only, it was not possible to distinguish dominant parental effects from domestication effects.

The current study aimed to expand existing knowledge of transcriptomic differences between wild and domesticated Atlantic salmon by investigating embryos for the first

time, and employing reciprocal hybrids to help dissect parental effects from the effects of domestication. Focusing on early life stages also has the benefit of minimizing environmental effects and the impact of early growth divergence on the transcriptome due to the fact that farmed salmon outgrow wild salmon by up to three fold by four months into feeding (Solberg et al., 2013a). The approach has inherent limitations, however. The microarray analysis is limited to the set of preselected probes on the platform. Analysis of whole embryo transcriptomes is likely to be relatively insensitive to differences in tissue-specific transcript expression, especially from smaller organs / low abundance cell types. For example, cellular signaling is employed by all cells regardless of the tissue of origin and as such its members are expressed across the body (Elliott and Elliott, 2009). Organism wide expression of cell signaling may promote the ability to detect this function when gene expression of whole individuals is studied. Finally, it is important to bear in mind that the current study utilized only one wild and one domesticated stock, thus some of the observed differences may be specific to these stocks and not necessarily solely due to a domestication effect. The remainder of this section comprises an examination of some of the key pathways identified, in order to provide biological context to the observed differences. Overall, the study identified pathways with *metabolic*, *immune* and *nervous system*, *genetic* and *environmental information processing* functions that displayed altered expression between the studied wild and domesticated Atlantic salmon embryos and builds upon earlier whole animal studies of transcriptomic responses to domestication in a number of fish species and life stages (Bicskei et al., 2014; Lanes et al., 2013; Mommens et al., 2014; Roberge et al., 2008, 2006).

4.4.1 Domestication is a form of adaptation

Domestication is possible because “organic beings” have the ability to adapt to the changing environment imposed upon them (Darwin, 1875b). As such one would expect relevant biological pathways involved in adaptation to a farm environment may

be differentially expressed between wild and domesticated fish. *Cell signaling* mediates responses to internal and external environmental cues and therefore may be affected by domestication. *Cell signaling* provides basal level control of cell replication, differentiation and apoptosis and the regulation of metabolic events, including receiving signals and responding to constantly altered physiological requirements. Control is achieved through the action of three broad signaling classes: neurotransmitter substances, hormones and cytokines or growth factors (Elliott and Elliott, 2009). In lower vertebrates, such as fish, cytokines and neuropeptides perform roles in both neuroendocrine and immune systems, including responses to stress (Nardocci et al., 2014; Tort, 2011). The process of fish domestication has been suggested to involve increased selection pressure on genes and pathways facilitating improved tolerance of acute and chronic stress, since individuals that perform better under farm conditions are more likely to be selected for broodstock (Øverli et al., 2005; Solberg et al., 2013a). As a key mediator of the stress response, modulation of cellular signaling is likely to be involved in the process of domestication. This was evident in the present study with detection of differential expression of stress-associated nervous and endocrine pathways between the two stocks. In particular, the *glutamatergic synapse* pathway was found to differ in this study and a previous study of wild vs. domesticated Atlantic salmon fry (Bicskei et al., 2014). Changes in this pathway have been associated with domestication in pigs, where expression of glutamate receptors affecting neural control of eating behaviours was shown to be linked to tameness (Moon et al., 2015). In addition, two pathways linked with domestication in birds (Nätt et al., 2012) were prominent in the current study; *long-term potentiation* which has a role in memory consolidation (Wei et al., 2012) and *GnRH signaling*, master regulator of vertebrate reproduction (Onuma et al., 2011).

4.4.2 Potential trade-offs between immune function and growth

In addition to pathways involved in adaptation to the farm environment, domestication may also affect those which enhance farm traits important to broodstock selection, in particular increased growth.

4.4.2.1 Up-regulated mRNA translation

Mitogen activated protein kinase (MAPK) signaling, as well as its role in responding to stress, also regulates *mRNA translation* and classical *MAPK signaling* promotes protein synthesis (Carriere et al., 2011). Thus selection for improved growth traits in domesticated fish may explain why *MAPK signaling* pathways were enriched in this study. *MAPK signaling* pathways have also been shown to be affected by domestication in birds (Nätt et al., 2012) and mammals (Amaral et al., 2011; Park et al., 2014; Yang et al., 2014). Up-regulation of *ribosome* and *RNA transport* pathways in the domesticated salmon embryos may also reflect processes which can enhance growth. Ribosomes are the site of protein synthesis, which is principally regulated at the translation initiation stage, allowing plasticity of expression. The differential expression of translation initiation factors 3E and 5 and large ribosomal subunit 6 and 7, identified in the current investigation, are similarly involved in this step (Jackson et al., 2010). Genes involved in protein synthesis, and hence growth, have been reported to be over represented in comparisons of transcript expression between wild and domesticated salmonid stocks (Bicskei et al., 2014; Devlin et al., 2009; Roberge et al., 2006; Sauvage et al., 2010; White et al., 2013) and between fast and slow growing rainbow trout (*Oncorhynchus mykiss*) (Xu et al., 2011). Xu *et al.* (2011) concluded that up-regulation of these corresponded to the earlier onsets of developmental processes in fast-growing families, as early as 15 days post fertilisation. Although increased protein synthesis, thus growth is indicated by the molecular data presented here, growth divergence of the Figgjo and Mowi strains has only been macro-phenotypically evident post first feeding (Solberg et al., 2014).

4.4.2.2 Up-regulated metabolic pathways

During early development, the embryo relies on its yolk sac to provide nutrients to sustain its growth and survival. This includes yolk lipids, the source of essential fat-soluble vitamins and triacylglycerol, as well as cholesterol, a required component of cell signaling molecules, membrane components, and sources of fuel (Anderson et al., 2011). Many of the digestive functions observed in hatched fry are already present in embryos at the time of sampling in this study (Vernier, 1969). Several pathways of the *lipid, carbohydrate and energy metabolism* functions were found to be up-regulated in the domesticated Atlantic salmon embryos, as well as in feeding fry (Bicskei et al., 2014), although specific pathways differed, possibly due to differences in processes involved in metabolizing yolk deposits and external food. For example, carbohydrate metabolism pathways differentially regulated between wild and domesticated in the embryos are involved in *fructose*, *mannose* and *galactose metabolism*, whereas *glycolysis/gluconeogenesis* and *propanoate metabolism* pathways were identified in the feeding fry life-stage. *Sphingolipid metabolism* was detected as a differentially expressed lipid metabolism pathways in the embryo stage, whereas *fatty acid degradation* and *elongation* and *glycerolipid metabolism* were found to be differentially perturbed in the feeding fry stage. Indeed, the activation of the glycolytic and fatty acid pathways is associated with gene expression changes occurring during the transition from endogenous to the exogenous feeding of fish (Mennigen et al., 2013).

4.4.2.3 Down-regulation of immune genes

Cell signaling is particularly important during embryonic development (Yang et al., 2013), with reciprocal gene regulation in both directions being characteristic of these regulatory pathways (Luo et al., 2009). Major overlaps between members of signaling and immune pathways may mask the direction of change of immune pathways. For this reason expression of some key genes was investigated, including representatives of different groups of cytokines; four chemokines (CCLs and CXCLs), three tumour

necrosis factor (TNF) ligands/receptors, and an interferon α (IFN-α). The majority of these showed lower expression in domesticated embryos compared to wild counterparts. Chemokines and TNFs play a pivotal role in immune function, but some members are also involved in stress responses and developmental processes (Alejo and Tafalla, 2011; Ottaviani and Franceschi, 1996; Wiens and Glenney, 2011). It has previously been proposed that domestication in salmonids may have resulted in immunosuppression, due to a trade-off between growth and immune function (K. A. Glover et al., 2006). In addition, since domesticated fish may in general display higher stress-tolerance, it has also been suggested that immune genes might be collaterally selected during domestication (Øverli et al., 2005; Solberg et al., 2013a).

Two cytokines; C-C motif chemokine 19 (CCL19) and TNFR superfamily member 5 (TNFRSF5) were not down-regulated, as expected from the above, in domesticated fish. CCL19 is referred to as a homeostatic or dual function chemokine (Peatman and Liu, 2007) and has been implicated in embryonic axis formation in zebrafish (Wu et al., 2012). Hence it may be more significant in a developmental than in an immune role. TNFRSF5 does not play a role in any of the significantly differentially expressed immune pathways occurring only in signaling pathways. It is noteworthy that, interferon regulatory factor 7 (IRF7), a transcription factor known to regulate IFN-α genes (Marié et al., 1998) and down-regulated in domesticated embryos in this study, has been proposed as a marker for assessing egg quality in Atlantic halibut (*Hippoglossus hippoglossus*) and is associated with hatching success (Mommens et al., 2014).

4.4.3 Organogenesis

Two *cell communication* pathways and the *cell adhesion molecules* pathways were found to be differentially expressed between wild and domesticated Atlantic salmon embryos but not in sac or fed fry (Bicskei et al., 2014), this reflecting life stage specific differences between stocks. These and several differentially expressed signaling pathways identified (not necessarily unique to embryos), are all known to participate in

organ development. For example, the *Hippo signaling* pathway, found to be differentially expressed between wild and domesticated stocks is involved in determining organ size and mediates crosstalk with other pathways (Halder and Johnson, 2011). NF-KB/IKB proteins, in addition to their immune function, have also been shown to be vital for organogenesis, e.g. zebrafish notochord development (Correa et al., 2004). The *wnt signaling* pathway which is responsible for tissue morphogenesis, was also found to be up-regulated in domesticated Atlantic salmon sac fry (Bicskei et al., 2014). According to Steinberg's differential adhesion hypothesis the basis of organ self-assembly is the segregation of cells with similar adhesive properties to achieve the most thermodynamically stable pattern (Clevers et al., 2014). As such WNT proteins and cellular communication/cell adhesion pathways, are closely linked (Rao and Kühl, 2010) and were also identified as differentially expressed in the current study. Sphingolipids, and their more complex, glycosylated derivatives, glycosphingolipids, as well as being components of cell membranes are also involved in cell signaling and adhesion (Lahiri and Futerman, 2007) In line with this, *glycan* and *lipid metabolism* pathways were up-regulated in domesticated embryos. The epithelial–mesenchymal transition (EMT) is a process in which tightly adjoined basal polarity epithelial cells acquire migratory mesenchymal properties (Lamouille et al., 2014). This process involves most of the differentially expressed *signaling* and *cellular communication* pathways identified in this study, including *MAPK, NF-kappa B, and wnt signaling, cytokine-cytokine receptor interactions, ECM-receptor interactions, cell and focal adhesion and gap junction.* EMT, in its developmental role, is involved in organ development and neural crest cell migration (Kalluri and Weinberg, 2009). Although changes occurring during neural crest development through domestication have been suggested to provide an explanation for some of the common inter-species similarities of domesticated animals (Wilkins et al., 2014), involvement in organ development fits the sampling timeline better. Sampling took place after eyeing of embryos, which occurs in the last third of embryogenesis. This phase of development

is characterized by organogenesis; the appearance of fins and formation of the internal organs and circulatory system. Eyeing occurs in stage 24 of salmonid development, whereas the neural tube is considered to be formed by stage 14 (Velsen, 1980; Vernier, 1969).

4.4.4 Parental effects on gene expression

In genes found to be significantly differentially expressed between pure crosses, hybrid gene expression ranged from intermediate to fully polarized towards expression of one or other parent. Hierarchical clustering revealed that the behaviour of a number of genes in hybrid fish reflected that of the maternal parent (wild or farmed). Within this group there was a high abundance of cytochrome related genes, involved in oxidative phosphorylation (mitochondrial subunit/precursors of the cytochrome b-c1 complex subunit 6 and cytochrome c oxidase subunit 4 isoform, NADH dehydrogenase 1 subunit C2 and NADH dehydrogenase iron-sulfur protein 7 and an ATP synthase) and metabolism of xenobiotics (microsomal glutathione s-transferase 1 and 3 and cytochrome P450, family 2, subfamily D). These processes have also been reported as having been affected by domestication in a number of fish species including brook charr (*Salvelinus fontinalis*), Atlantic salmon and Atlantic cod (*Gadus morhua*) (Debes et al., 2012; Lanes et al., 2013; Sauvage et al., 2010). In addition, Crockford (Crockford, 2006, 2004, 2003) proposed that domestication is the product of heterochrony, *i.e*. changes in developmental rates and/or timing, induced by thyroid hormone altered oxidative reaction and metabolism rates involving carbohydrates and lipids in particular. Two haemoglobin subunits were also differentially regulated between salmon embryos and clustered with genes that showed maternal influence (Figure 4.2 and 4.3). Haemoglobin genes have previously been identified as differentially regulated transcripts between multiple wild and domesticated brook charr reciprocal hybrids, suggesting consistent parental effects (Bougas et al., 2013a).

In hybrids, maternal effects were predominant for many genes. Maternal effects are recognized as being of particular prominence in the embryonic stage of fish (Kamler, 2007; Tadros and Lipshitz, 2009), however there is a growing body of evidence demonstrating paternal contributions (Jiang et al., 2013). The great majority of differences in gene expression between hybrid and pure crosses were common to both reciprocals. These shared differences were more likely to show dominance with respect to the origin of the mother rather than the origin of the stock, indicative of maternal dominance. It was noted that differential expression of wild♀ × domesticated♂ hybrids showed slightly higher combined dominance (42% vs 32.1%) and lower additivity (33.3% vs 42.3%) than domesticated♀ × wild♂ hybrids (Table 4. 2). In line with these results, Bougas *et al.* (2013) highlighted the relevance of additivity (54.3%) and the importance of maternal effects (40%) when comparing the gene expression inheritance of wild-domesticated brook charr hybrids (Bougas et al., 2010). However, data from the current study suggest that over-dominance may be more pronounced at the embryo stage (Table 4. 2). In addition, although similar results were published for the F1 generation of dwarf and normal lake whitefish hybrids, over-dominance increased in backcross hybrids at the expense of additivity (Renaut et al., 2009).

4.4.5 Implications for interactions between wild and farmed salmonids

Escape from commercial farms, and genetic interactions with wild conspecifics, represents one of the major environmental challenges to a sustainable Atlantic salmon aquaculture industry (Taranger et al., 2015). Each year hundreds of thousands of farmed Atlantic salmon escape into the wild. While many of these disappear, some enter rivers (Fiske et al., 2006; Youngson et al., 1997), and, genetic changes in wild populations as a result of farmed salmon interbreeding has been observed in Ireland and Norway (Clifford et al., 1998; Crozier, 1993; Glover et al., 2012, 2013; Skaala et al., 2006). This has caused significant international concerns over the long-term fitness

of wild populations given that wild salmon populations may display local adaptations to the rivers they inhabit (Garcia de Leaniz et al., 2007), and that the offspring of farmed salmon display reduced survival in the wild compared to the offspring of wild salmon (Fleming et al., 2000; McGinnity et al., 2003, 1997; Skaala et al., 2012). However, at present, the underlying genetic differences between domesticated and wild Atlantic salmon are still somewhat vague but see (Besnier et al., 2015), despite studies such as the present. It is therefore important that further studies are conducted in order to fully elucidate the genomic differences between farmed and wild Atlantic salmon.

4.5 Conclusion

The results of this study have highlighted the effects of Atlantic salmon domestication on *signaling*, *immune* and *mRNA translation* pathways. Although, in the absence of tissue specificity, results are more difficult to interpret, processes that affect most cells types can still be identified, regardless of the life stage and tissue distribution. *Cell signaling* combined with *cell communication* and *adhesion* pathways may be particularly relevant in the context of the developing embryo and in organogenesis in particular. Since some of the perturbed *signaling* pathways have also been detected in later life stages, and together with the identified *nervous system* pathways are often affected by external stimuli, these differences could have arisen through adaptation to human controlled farm environment. Increased *mRNA translation*, due to its link to protein synthesis, could be considered a logical outcome of selection for growth. With the aid of reciprocal hybrids this study has allowed separation of heritability of domestication effects and those due to parental effects. In particular, this study has shown the importance of maternal effects in wild-domesticated hybrids, and highlighted the relatively high percentage of over-dominant gene expression that may be typical of the embryo stage. An important consideration is that this study compared the transcriptome of a single wild and domesticated strains. As such, to draw general conclusions regarding the outcome of the genetic interactions between wild and domesticated fish, support from future studies of multiple strains is required.

Chapter 5 - Transcriptomic comparisons of communally reared wild, domesticated and hybrid Atlantic salmon (*Salmo salar* L.) fry under stress and control conditions

5.1 Background

Domestication is possible because many organisms have the ability to rapidly adapt to the human-modified environment (Darwin, 1875a). The two different environments that wild and domesticated fish experience thus exert different selection pressures and engender specific local adaptations (Price, 2002). Domestication is beneficial to humans, and advantages are achieved via both intentional selection for specifically desired traits and through passive selection for traits that improve fitness under a culture environment. In the case of the Atlantic salmon, *Salmo salar* L. , specifically selected traits largely comprise economically important production traits including increased growth, late maturation, greater disease resistance and improved flesh quality (Gjedrem, 2010, 1975). Simultaneously, unintentional selection takes place through co-selection of traits via genetic linkage and through local adaptation, via natural selection, to the human-controlled environment. For example, adaptations to captivity, such as performing well in human presence and under high stocking densities or efficiently metabolising fish feed, improve fitness under farm conditions. Other traits such as those mediating predator-avoidance or foraging behaviour, that are essential to survival in the wild, lose significance in aquaculture (Price, 2002). Because the fitness consequences of genetically controlled traits shift during domestication, the optimal investment of resources differs between farm and wild niches. According to resource-allocation theory, since resources available for a given individual are limited, the increased energy demands of one trait have to be counterbalanced by reducing energy allocation to other, at least momentarily, less important traits. Since growth is often under strong selection in domesticated populations, while at the same time immune function is both necessary and highly

energy demanding, an important trade-off between growth and immune function has been proposed to occur for domesticated animals, in particularly for species selected for increased production traits (Rauw, 2012).

Due to the protected / simplified environment of captivity and reliance upon humans to meet key needs, reduced environmental awareness has also been proposed as a consequence of the process of domestication. This may occur through the decline of information acquisition and transmission systems, such as sensory organs and synaptic activity. Environmental awareness is an evolutionarily highly important trait in the wild, but its reduction is likely to be beneficial for domesticated species through reduction of stress (Hemmer, 1990). The effect of domestication upon complex traits can be extremely difficult to disentangle such that the activity of traits with multiple biological functions may be enhanced in one species, due to a certain beneficial function, but decreased in another, due to a different function that bears more weight for that organism. As a result, and in contrast to the hypothesized benefit of reduced synaptic activity in domesticated animals, enhanced excitatory synaptic plasticity and its contribution through enhanced memory and learning to effective interaction with humans has been proposed in dogs (*Canis familiaris*) (Li et al., 2014).

Response to stimuli, including stress, is heavily context-dependent and among other factors it is influenced by variability in individuals' experience of the stimulus (Wendelaar Bonga, 1997). Wild and domesticated fish are adapted to different rearing environments and among other traits, their stress responsiveness also differs (Gross, 1998). In a study of growth reaction norms, reduced responsiveness to chronic stress was demonstrated for the domesticated Atlantic salmon strain studied here, when it was compared to the offspring of wild fish under hatchery conditions (Solberg et al., 2013a). Because stress disturbs homeostasis and its restoration is energy demanding, increased stress-responsiveness requires an increased allocation of available resources. To cover this demand, energy is generally directed away from functions

that are non-vital and have high energetic costs associated with them, such as growth and reproduction (Wendelaar Bonga, 1997), necessitating a further trade-off under culture conditions.

Alteration of gene expression provides a rapid and plastic response to stress (Holcik and Sonenberg, 2005; Yamasaki and Anderson, 2008). In addition, since gene expression reflects evolutionary change (Carroll, 2005; King and Wilson, 1975), it is suitable for studying the process of domestication. The Atlantic salmon transcriptome is known to be affected by domestication (Roberge et al., 2008, 2006) and some changes are likely to be life-stage dependent (Bicskei et al., 2015, 2014). The aim of this study was i) to investigate the functional significance of transcriptomic differences between wild and domesticated Atlantic salmon fry under control and acute stress conditions, ii) to identify any existing stock-specific transcriptomic stress responses resulting from gene × cross interactions and iii) to determine the mode of heritability of the genes identified as differentially expressed under control and stress conditions. The elucidation of genetic differences and interactions between wild and domesticated Atlantic salmon populations will help to predict the consequences of introgression of genes into the wild salmon gene pool from domesticated escapees and to better understand the process of domestication in the context of the Atlantic salmon genome.

5.2 Methods

5.2.1 Biological samples

The domesticated broodstock used in this study originated from the Norwegian Mowi strain. This commercial strain has been maintained in culture for over 10 generations and has been selected for a range of commercially important traits. In experimental comparison with wild populations, this domesticated strain has been previously demonstrated to display significantly higher growth rates under hatchery conditions (Glover et al., 2009; Solberg et al., 2013a, 2013b), and lower survival in the wild

(Skaala et al., 2012). Wild adult broodstock originated from the Figgjo River in south west Norway. These fish were confirmed as born in the wild based upon reading their scales (Lund and Hansel, 1991). This aquaculture strain and wild populations are described in greater detail elsewhere (Bicskei et al., 2014).

Both domesticated and wild broodstock were simultaneously stripped for gametes and crosses were established on 23rd November 2011. Originally three sets of crosses were established each set comprising four combinations of crosses from three wild (W) and domesticated (D) parents (i.e. pure wild, W♀W♂; pure domesticated, D♀D♂; and reciprocal hybrids W♀D♂ and D♀W♂). Although three families per cross were created, reared and sampled, a possible farm escapee was identified through scale reading among the wild fish, and thus the microarray experiment was restricted to two families per cross from the laboratory work onwards. Adipose fin samples from the parents and caudal fin samples from the offspring were retained for DNA profiling. Fertilised eggs were reared under standard hatchery conditions in single family incubators at ambient temperature (4.2-8.1℃). At the eyed egg stage on 2nd February 2012, families and crosses were mixed to generate four replicate pools; 30 individuals per family per set of crosses (i.e. 4 pools of 360 eggs). These were then reared in four compartments within the same tank. On 28th March 2012 hatched fry were transferred into individual heated tanks (13℃, 1m^3, 45 cm water depth) to initiate exogenous feeding and construct duplicate experimental groups comprising two control and two treatment tanks. Fry were fed on standard hatchery diet 24hr a day by automatic feeders.

0n 17th April 2012, 3 weeks post swim-up (c. 985°d post-fertilization) feeding in all tanks was stopped. Water levels in the duplicate experimental tanks were altered over a 24h period; 3 hours at low depth (2.5 cm) followed by 3 hours at normal depth (45 cm) – repeated 4 times. Levels were altered over a 15 min period. In addition to crowding, at low water level the fish experienced increased water splashing from the

inlet feed and increased current velocities. As a first response fish broke schooling structure and were distributed randomly in the tanks. After approximately 20 minutes more structured swimming was observed and fish became responsive to human presence, which was not the case in the initial phase. After 24 hr fish from all four tanks were euthanized with metacaine (Finquel® Vet, Scanvacc, Årnes, Norway), and transferred immediately into an RNA stabilisation buffer (3.6 M ammonium sulphate, 18 mM Sodium Citrate, 15 mM EDTA, pH 5.2). After 24 h incubation at 10°C in this buffer the fry were removed and stored at -70^{o}C until homogenized.

The experiment was conducted in accordance with Norwegian regulations for the use of animals in research. The experimental protocol was approved by the Norwegian Animal Research Authority (NARA) (Norwegian research permit 4368).

5.2.2 Family assignment

To assign individual fish sampled from all of the four experimental tanks to families, microsatellite genotyping was performed. A total 846 fish was genotyped to achieve 24 individuals from each family and from both conditions (control and stress); since tanks were duplicated, this meant 12 individual per family and per control or stress tank. DNA was extracted from tail samples in 96 well plates using a Qiagen DNeasyW96 Blood & Tissue Kit following manufacturer's instructions. Five microsatellite loci were amplified in one multiplex PCR; SsaF43 [GenBank: U37494], Ssa197 [GenBank: U43694.1], SSsp3016 [GenBank: AY372820], MHCI (Grimholt et al., 2002) and MHCII (Stet et al., 2002), PCR products were run on an ABI 3730 Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and size-called according to the 500LIZ™ standard. Genotypes were identified using GeneMapper V4.0 (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and family assignment was performed via FAP; Family Assignment Program v3.6 (Taggart, 2007). Only individuals unambiguously assigned to families were used in subsequent analysis.

5.2.3 Microarray Experimental Design

Microarray analysis was performed using a custom-designed, oligonucleotide microarray platform (Agilent) with four 44 K probe arrays per slide (Salar_3; ArrayExpress accession number A-MEXP-2400). The general design of the microarray has been described in detail elsewhere (Tacchi et al., 2011) and further used / validated in a number of subsequent studies (e.g. Morais *et al*. 2012; Martinez-Rubio *et al*. 2012; Bicskei *et al*. 2014; De Santis et al. 2015.).

Dual-label hybridisations were undertaken, with each experimental sample (Cy3 labelled) being competitively hybridised against a pooled reference control (Cy5 labelled) comprising equimolar amounts from each experimental RNA sample. The interrogations comprised 48 separate hybridisations; 4 cross types (pure wild, pure domesticated and reciprocal hybrids) x 2 conditions (stress and control) x 6 biological replicates (2 tank replicates, 3 samples per tank).

5.2.4 RNA Extraction and purification

Fry from only two of the three sets of crosses were subsequently used, as scale reading analysis raised a suspicion that one of the wild parents may have been a farm escapee. Whole fry (n = 384) were homogenised rapidly in Tri Reagent (Sigma–Aldrich®, St. Louis, U.S.A.) using a Mini-Beadbeater-24 (BioSpec Products Inc., Bartlesville, USA) and RNA extracted following the manufacturer's instructions. RNA quantity and quality were assessed by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.) and agarose gel electrophoresis respectively. For each biological replicate (hybridisation sample), equal amounts of total RNA from eight individuals per tank were pooled (four fry per family, two families per cross type) and then re-quantified and quality assessed as described above.

5.2.5 RNA amplification and labelling

Each pooled RNA sample was amplified (TargetAmpTM 1-Round Aminoallyl-aRNA Amplification Kit, Epicentre Technologies Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. Following quality control (Nanodrop quantification and agarose gel electrophoresis) each aRNA sample was indirectly labelled and purified. Briefly, Cy dye suspensions (Cy3 and Cy5) in sufficient quantity for all labelling reactions were prepared by adding 42 µL high purity dimethyl sulphoxide (Stratagene, Hogehilweg, The Netherlands) per tube of Cy dye (PA23001 or PA25001; GE HealthCare, Little Chalfont, Bucks, UK). Individual samples (2.5 µg aRNA in 10.5 µL H_2O) were denatured at 75°C for 5 min and then 3 µL 0.5 M $NaHCO_3$ pH8.5 and 1.5 µL Cy3 dye added. The reference pool consisted of the same proportions per sample, but 1 µL Cy5 dye was used to label 2.5 µg pooled aRNA. Samples were incubated for an hour at 25^{o}C in the dark, purified using an Illustra AutoSeq G-50 Dye Terminator Removal Kit (Qiagen GE Healthcare), and concentration, dye incorporation and purity were assessed via spectrophotometer (NanoDrop) with products also visualised on a fluorescent scanner (Typhoon Trio, GE Healthcare).

5.2.6 Microarray hybridisation and quality filtering

Hybridisation was performed over two consecutive days using the Agilent Gene Expression Hybridisation Kit (Agilent Technologies) as per manufacturer's instructions. For each reaction, 825ng Cy5 labelled reference pool and 825 ng Cy3 labelled individual samples were combined in 35 µL nuclease free water and then 20 µL fragmentation master mix added (11 µL of 10x blocking agent, 2µL 25x fragmentation buffer and 7µL nuclease free water). The reactions were then incubated at 60^{o}C in the dark for 30 mins, chilled on ice, and mixed with 57 µL 2x GEx Hybridisation buffer (pre heated to 37^{o}C), Following centrifugation (18000 x g for 1 min) the samples were kept on ice until loaded (103 µL) in a structured randomised order onto the microarray

slides. Samples from the six biological replicates were divided across different slides, Cy3 fluorescence content (dye incorporation rate x volume) was also taken into consideration. To aid scanning, samples with the most similar amounts of Cy3 were grouped on the same slide. Hybridisation was carried out in a rotating rack oven (Agilent Technologies) at 65^{o}C, 10 rpm over 17 hours.

Following hybridisation, slides were washed in Easy-DipTM slide staining containers (Canemco Inc., Quebec, Canada). First, a 1 min incubation at room temperature (c. 20°C) in Wash Buffer 1 was performed, with gentle shaking at 150 rpm (Stuart Orbital Incubator). Slides were briefly dipped into Wash Buffer 1 pre-heated to 31^{o}C, then placed into Wash Buffer 2 (31^{o}C) for 1 min at 150rpm. Finally, the slides were transferred to acetonitrile for 10secs and then Agilent Stabilization and Drying Solution for 30 secs. The slides were then air dried in the dark and scanned within three hours.

Scanning was carried out at 5µm resolution on an Axon GenePix Pro scanner at 70% laser power. The “auto PMT” function was enabled to adjust PMT for each channel such that less than 0.1% of features were saturated and so that the mean intensity ratio of Cy3:Cy5 signal was close to one. Agilent Feature Extraction Software (v 9.5) was used to identify features and extract background subtracted raw intensity values that were then transferred to GeneSpring GX (version 13.0) software where the quality filtering and normalisation steps took place. Intensity values ≤ 1 were adjusted to 1 and a Lowess normalisation undertaken. Stringent quality filtering ensured that features that represented technical controls, saturated probes, probe population outliers or probes which were not significantly different from the background were removed. Agilent feature extraction software was used to determine whether a probe was positive and significant based on a 2-sided t-test, indicating whether the mean signal of a feature was greater than the corresponding background. A probe was retained if it was positive and significant in at least 75% of the arrays in any 4 of the 8 experimental

groups. This process resulted in 30164 of the original 43413probes being considered eligible for downstream analysis.

Details of the microarray experiment have been submitted to ArrayExpress under accession number E-MTAB-3679. The recording of the microarray experimental metadata complies with Minimum Information About a Microarray Experiment (MIAME) guidelines.

5.2.7 Microarray data analysis

Three dimensional principal component analysis (3D-PCA) was performed in GeneSpring on all transcripts that passed quality filtering. The covariance analysis was done on the overall gene expression of individual samples, as part of quality control; testing for outlier samples as well as exploring the differentiation between and within replicates of experimental groups. The number of principal components was set to four (default) with the three principal components that explained the major trends of variation shown on the axes. This PCA is solely based on gene expression and independent of experimental grouping.

To investigate cross-specific stress response, differentially expressed transcripts were identified in GeneSpring using a 2-way ANOVA. Here, cross (wild, reciprocal hybrids, domesticated) and condition (stress and control) were considered as factors and multiple testing correction (Benjamini-Hochberg, $p<0.05$) was performed. The above statistical analysis was carried out on all four crosses and also separately, limited to the two pure crosses - excluding reciprocal hybrids.

KEGG-based functional analyses of cross- and condition-specific transcriptomic differences were achieved via two analytical approaches, both carried out in *R software v.3.1.3* (R Core Team, 2015). First, rank based *GAGE* analysis (Generally Applicable Gene-set/Pathway Analysis) (Luo et al., 2009) was performed, implementing Mann Whitney U tests, then the *romer* function from the *limma package*

(Linear Models for Microarray Data) (Smyth, 2004) was used to achieve more robust results, that are supported by different methods. For *GAGE* results a corrected *p-value* for significance of <0.1 was applied, whereas for *romer* the cut-off for the number of genes was 10 and *p-value* <0.05. For both techniques, a total of six contrasts were considered. First, to address the primary aim of the experiment, identifying functional differences between wild and domesticated stocks, the pure crosses were compared under control (Cross control) and stress conditions (Cross stress). Then, to identify responses to the stress treatment, stressed fish of wild and domesticated origin were compared to control fish from the corresponding crosses (Condition wild and Condition domesticated). Second, based on the number of differences detected by statistical approaches, the hybrids' contribution seemed to be considerable for the condition factor of 2-way ANOVA, and it was hypothesised that they may exhibit a strong response to the stressor. Therefore, the effect of stress was also investigated for the hybrid stocks (Condition WD and Condition DW). To achieve unique KO-probe association, where multiple probes were assigned to the same KO number, probes with the lowest overall p-value based on a 2-way ANOVA were chosen. Since pathways belonging to the human disease functional group are difficult to interpret in fish, this group was excluded from the gene enrichment analysis. The significant pathways jointly supported by both analyses are discussed.

To look at heritability of differentially expressed genes between the genetically divergent crosses, 1-way ANOVA (unequal variance) was performed with 5% FDR (Benjamini-Hochberg) and Student Newman-Keuls (SNK) *post-hoc* analysis using GeneSpring. To avoid repeated counting of the same gene, only transcripts that had KEGG annotation available were chosen and where multiple probes were present for the same gene, the probe with the highest significance was chosen. The obtained genes were assigned to the following heritability categories:

Maternal effect: differential expression between W♀W♂vs D♀W♂ or D♀D♂vs W♀D♂

Paternal effect: differential expression between W♀W♂ vs W♀D♂ or D♀D♂ vs D♀W♂

Parental effect: influenced by both maternal and paternal effects

Maternal only: unique to maternal effect

Paternal only: unique to paternal effect

For the unique differentially expressed genes obtained, additivity; α = (wild-domesticated)/2 and dominance parameters; δ = (wild + domesticated)/2-hybrid were calculated from normalised intensity values and α and δ/α were plotted using the ggplot2 package (Figure 5.3) (Wickham, 2009). Considering that, by definition, a transcript whose expression value in hybrids corresponds to the mid-value of the parents' is additive (i.e.: perfect additivity: δ/α = 0) and that a transcript whose hybrid gene expression value resembles more closely one parent or another is dominant (i.e.: domesticated dominance: δ/α = 1 and wild dominance: δ/α = -1), by halving the intervals we can presume that transcript expression corresponds to:

- additivity if $-0.5 < \delta/\alpha < 0.5$
- wild dominance if $-1.5 < \delta/\alpha < -0.5$
- domesticated dominance if $0.5 < \delta/\alpha < 1.5$
- over-dominance if δ/α falls out of the interval −1.5-1.5.

For ease of plot interpretation, genes with $|\delta/\alpha|>5$ were excluded from the scatter graph but were considered in the heritability table.

The function and expression profiles of genes that were likely to be responsible for the differences detected in the heritability patterns of the reciprocal hybrids in response to stress were further investigated. According to the heritability analysis, maternal effects were considerably more pronounced in the stressed W♀D♂ hybrids than in the stressed D♀W♂, and this seemed to occur at the expense of additivity (Table 5.5).

Genes that were wild dominant in the former, and additive/wild dominant in the latter stressed hybrid cross were selected, annotated (KEGG), subjected to hierarchical clustering (Pearson correlation) using the *heatmap.2* function of the *gplots R* package (Warnes et al., 2014) and presented on a heatmap.

5.3 Results

5.3.1 Expression data overview

3D-PCA clustered the samples according to condition (stress / control) and cross (wild / reciprocal hybrids / domesticated) (Figure 5.1). Pure wild and pure domesticated crosses were found to be the most divergent, whereas reciprocal hybrids were situated in between; suggesting an overall intermediate gene expression. Moreover, the positioning of the hybrids were indicative of their maternal origin, such as wild dam hybrids tended to be closer to pure wild crosses, while hybrids of domesticated dams clustered towards pure domesticated samples (Figure 5.1).

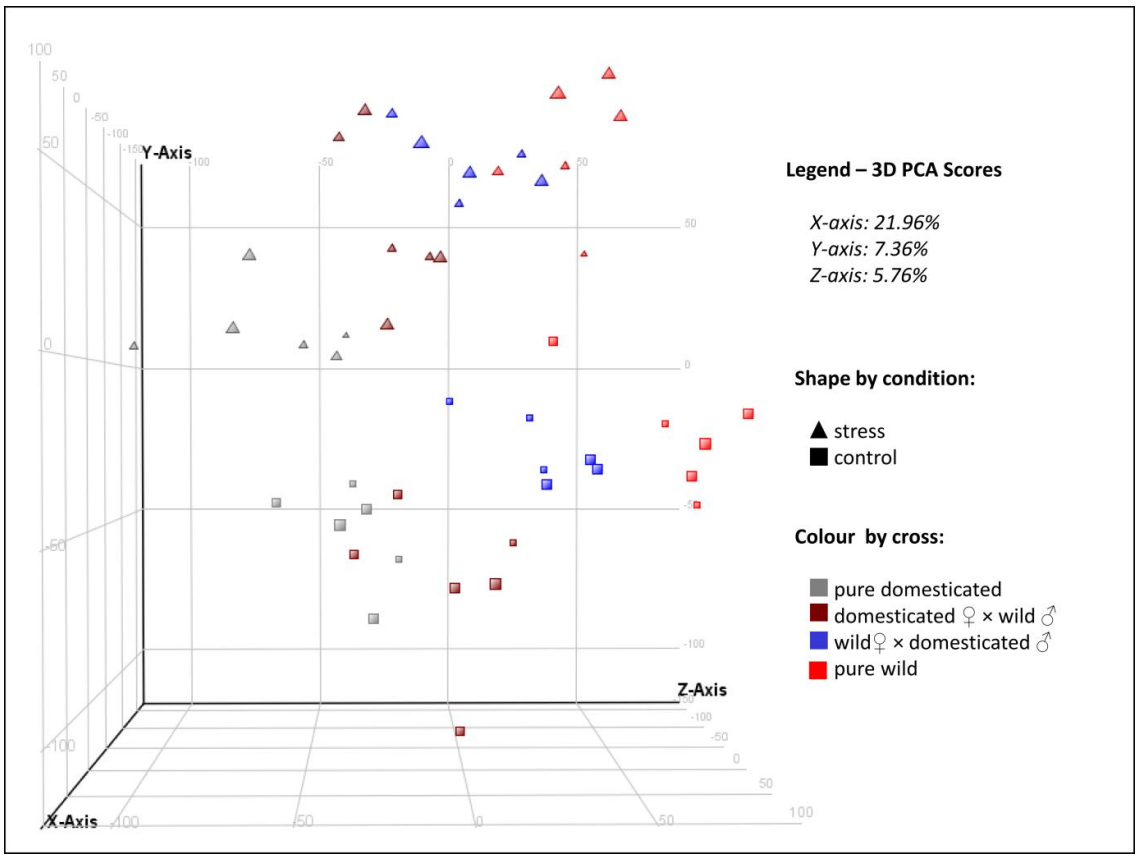
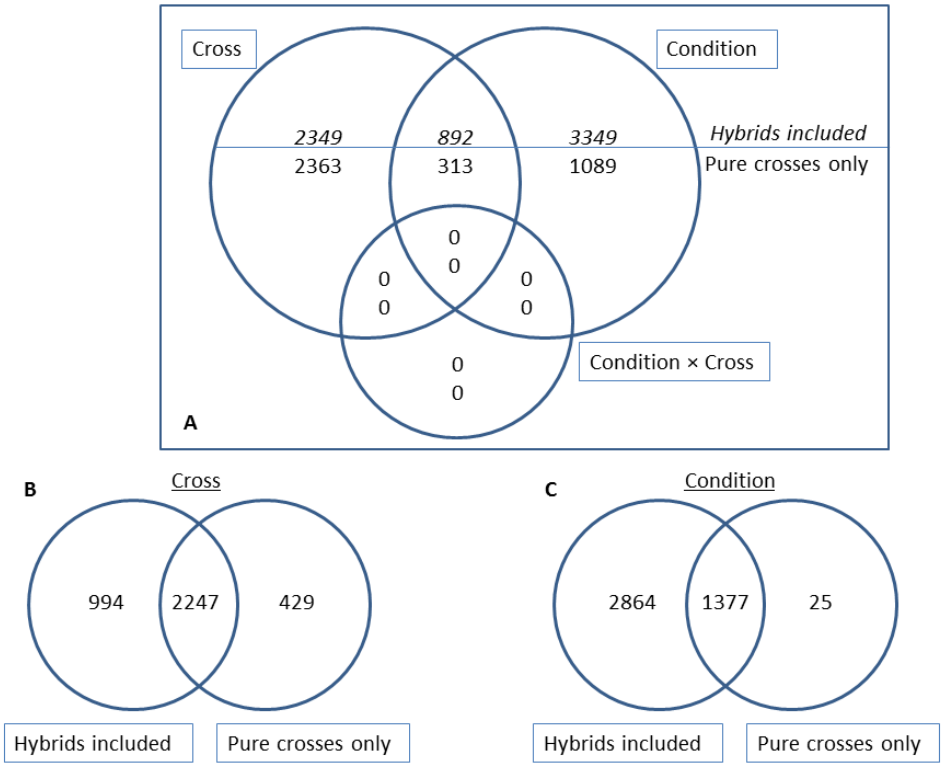


Figure 5.1 A 3-D representation of the PCA performed on all transcripts that passed quality filtering. Samples are colour coded by the experimental factors.

Statistical analysis (2-way ANOVA, FDR corrected $p<0.05$) revealed a number of differentially expressed transcripts among crosses and conditions, but no interaction between these two factors was detected (Figure 5.2A). Separate analyses were performed i) comparing pure wild and domesticated crosses only, and ii) considering all four crosses *i.e*. including reciprocal hybrids. Looking at the differential expression explained by cross (Fig 2B), the majority of transcripts (2247) were common to both analyses. In contrast, despite 1377 differentially expressed transcripts being common to both analyses for the factor condition, inclusion of hybrids provided a substantial addition of 2864 unique transcripts (Figure 5.2C).

Figure 5.2 A representation of the number of differentially expressed transcripts based on a 2-way ANOVA. **A.** Transcriptomic differences arising through variation between all crosses (WxW, WxD, DxW, DxD) conditions (stress and control) and the interaction of these two factors. The top numbers reflect statistics for all crosses including the hybrids, whereas the bottom numbers were generated by limiting the 2-way ANOVA to pure crosses only. **B.** The common and unique differences in cross-specific expression with and without consideration of reciprocal hybrids. **C** The common and unique differences arising from exposure to stress vs control conditions and detected with and without consideration of hybrids.



5.3.2 Functional analysis

Functional analyses of the transcriptomic differences between domesticated and wild stocks, as well as in response to stress were performed using two different software packages. Results are presented in Tables 5.1-5.3. Differences detected in pure cross domesticated origin fish relative to wild origin fish included down-regulation of *signal transduction* and *immune and nervous systems,* up regulation of *mRNA translation, carbohydrate metabolism* and *lipid metabolism* and *digestive system* and both up and down regulation of some pathways of the *endocrine system* (Table 5.1). Some of the differentially expressed biological functions were represented by a smaller number of pathways under stress conditions, the most pronounced being the *digestive system*, as a consequence of *protein and vitamin digestion and absorption* and *mineral absorption pathways* only being significantly different under control conditions.

In contrast to the above results, pathways differentially expressed in wild and domesticated pure crosses in stress relative to control conditions were less consistent (Table 5.2). Common transcriptional responses to stress, applicable to both pure crosses, included down-regulation of *cell growth and death* and *DNA replication and repair*. In addition, up-regulated *digestive and endocrine systems* appeared to be characteristic of the wild stress response, whereas up-regulated *signalling molecules and interaction* pathways were only found in domesticated fish.

Pathways differentially expressed between the stress and control states for hybrids showed some variation according to the direction of the cross (Table 5.3). Pathways that were consistent between both hybrids included down-regulation of *cell growth and death*, *DNA replication and repair* and up-regulation of *carbohydrate* and *lipid metabolism* in response to stress. In addition, up-regulation of *signal transduction* and *nervous system* pathways appeared to be D♀W♂ hybrid specific stress. Also, up-regulated *digestive* and *endocrine systems* were represented by a larger number of pathways in this hybrid, than in the W♀D♂ hybrid.

Table 5.1 Pathways found to be differentially expressed between wild and domesticated stocks under control and stress conditions by both gage and romer packages. The direction of change shown describes the expression of the pathway in the domesticated fish relative to wild counterparts. The terms “2D” and “Mixed” are used to describe pathways in which genes showed bidirectional change. “Genes” refers to the number of genes included in the gene set test.

				Control		Stress	
KEGG group	**KEGG sub-group**	**Pathway**	**Genes**	**gage**	**romer**	**gage**	**romer**
Cellular Processes	Cell communication	Focal adhesion	98	2D	Down	2D	Down
		Gap junction	39			2D	Down
	Cell growth and death	Cell cycle – yeast	54	Up	Up	Up	Up
	Transport and catabolism	Phagosome	76	Down	Down/Mixed	Down	Down/Mixed
		Endocytosis	105	Down/2D	Down	Down/2D	Down
		Peroxisome	54	Up	Up	Up	Up
Environmental Information Processing	Membrane transport	ABC transporters	27			Up	Mixed
	Signal transduction	MAPK signaling pathway	110	2D	Down	2D	Down
		NF-kappa B signaling pathway	64	2D	Down		
		Jak-STAT signaling pathway	56	2D	Down		
		Calcium signaling pathway	72	2D	Down	2D	Down
		PI3K-Akt signaling pathway	149	2D	Down/Mixed	Down/2D	Down
		VEGF signaling pathway	28			2D	Down
	Signaling molecules and interaction	Cell adhesion molecules (CAMs)	64	2D	Down/Mixed	2D	Down
		Neuroactive ligand-receptor interaction	112	Down/2D	Down		
		Cytokine-cytokine receptor interaction	94	Down/2D	Down	2D	Down
Genetic Information Processing	Replication and repair	DNA replication	33			Up	Up
	Transcription	RNA polymerase	27	Up	Up	Up	Up
		Spliceosome	109			Up	Up
	Translation	Ribosome biogenesis in eukaryotes	64	Up	Up	Up	Up/Mixed
		RNA transport	111	Up	Up	Up	Up/Mixed
		Ribosome	118	Up	Up/Mixed	Up	Up/Mixed
Metabolism	Amino acid metabolism	Arginine and proline metabolism	36	Up	Up		
	Carbohydrate metabolism	Amino sugar & nucleotide sugar metabolism	35	Up	Up/Mixed	Up	Up/Mixed
		Galactose metabolism	16	Up	Up/Mixed	Up	Up/Mixed
		Fructose and mannose metabolism	19	Up	Up/Mixed	Up	Up/Mixed
		Glycolysis / Gluconeogenesis	30	Up	Up/Mixed		

	Lipid metabolism	Sphingolipid metabolism	21	Up	Up	Up	Up
		Biosynthesis of unsaturated fatty acids	15	Up	Up/Mixed	Up	Up/Mixed
		Glycerolipid metabolism	25	Up	Up/Mixed		
		Primary bile acid biosynthesis	12	Up	Up/Mixed	Up	Up/Mixed
	Nucleotide metabolism	Pyrimidine metabolism	73	Up	Up/Mixed	Up	Up
	Xenobiotics biodegradation and metabolism	Drug metabolism - cytochrome P450	11	Down	Down/Mixed	Down/2D	Down/Mixed
Organismal Systems	Circulatory system	Vascular smooth muscle contraction	55	2D	Down	Down/2D	Down
	Development	Osteoclast differentiation	69	2D	Down	2D	Down
		Axon guidance	52	Down/2D	Down		
	Digestive system	Protein digestion and absorption	40	2D	Mixed		
		Vitamin digestion and absorption	17	2D	Up/Mixed		
		Mineral absorption	25	Up	Up/Mixed		
		Fat digestion and absorption	19	Up	Up/Mixed	Up	Up/Mixed
		Bile secretion	39	Up/2D	Up/Mixed	Up/2D	Up
		Salivary secretion	32			Down/2D	Down
	Endocrine system	Ovarian steroidogenesis	22	2D	Down	2D	Down
		Thyroid hormone synthesis	33	2D	Up/Mixed		
		PPAR signaling pathway	42	Up	Up		
		Insulin secretion	37			2D	Down
	Environmental adaptation	Circadian entrainment	45	2D	Down	Down/2D	Down
	Immune system	T cell receptor signaling pathway	61	2D	Down		
		Fc epsilon RI signaling pathway	29	2D	Down	2D	Down/Mixed
		B cell receptor signaling pathway	39	2D	Down	Down/2D	Down
		Complement and coagulation cascades	56	Down	Down/Mixed	Down	Down/Mixed
		Hematopoietic cell lineage	38	Down/2D	Down/Mixed	Down	Down
		Chemokine signaling pathway	88	Down/2D	Down/Mixed	Down/2D	Down/Mixed
		Natural killer cell mediated cytotoxicity	40	Down/2D	Down/Mixed	Down/2D	Down
	Nervous system	Glutamatergic synapse	50	2D	Down	Down/2D	Down
		Synaptic vesicle cycle	35	Down	Down		
		Serotonergic synapse	49	Down	Down	Down	Down
		Retrograde endocannabinoid signaling	46			2D	Down
	Sensory system	Phototransduction	14	Down	Down		

Table 5.2 Pathways found to be differentially expressed between control and stress conditions in pure wild and domesticated stocks by both gage and romer packages. The direction of change shown describes the expression of the pathway in the stressed fish relative to the control state. The terms “2D” and “Mixed” are used to describe pathways in which genes showed bidirectional change. “Genes” refers to the number of genes included in the gene set test.

KEGG group	KEGG sub-group	Pathway	Genes	Wild		Domesticated	
				gage	romer	gage	romer
Cellular Processes	Cell communication	Gap junction	39			2D	Down
	Cell growth and death	Cell cycle	88	Down	Down/Mixed	Down	Down/Mixed
		Cell cycle – yeast	54	Down	Down/Mixed	Down	Down/Mixed
		Meiosis – yeast	41	Down	Down/Mixed	Down	Down/Mixed
	Transport and catabolism	Endocytosis	105			2D	Up
Environmental Information Processing	Signal transduction	Hippo signaling pathway – fly	29	2D	Down	2D	Down
	Signaling molecules and interaction	Cytokine-cytokine receptor interaction	94			2D	Up
		Neuroactive ligand-receptor interaction	112			Up/2D	Up
Genetic Information Processing	Folding, sorting and degradation	Proteasome	40	Down	Down	Down	Down/Mixed
	Replication and repair	Base excision repair	28	Down	Down/Mixed	Down	Down/Mixed
		DNA replication	33	Down	Down/Mixed	Down	Down/Mixed
		Fanconi anemia pathway	35	Down	Down/Mixed		
		Homologous recombination	20	Down	Down/Mixed	Down	Down
		Mismatch repair	18	Down	Down/Mixed	Down	Down/Mixed
		Nucleotide excision repair	35	Down	Down/Mixed		
	Transcription	Spliceosome	109	Down	Down	Down	Down
	Translation	Ribosome biogenesis in eukaryotes	64	Down	Down/Mixed	Down	Down/Mixed
Metabolism	Carbohydrate metabolism	Glycolysis / Gluconeogenesis	30	Up	Up/Mixed		
	Energy metabolism	Oxidative phosphorylation	105	Up	Up	Up	Up
	Metabolism of cofactors and vitamins	Nicotinate and nicotinamide metabolism	12	Up	Up/Mixed		
	Nucleotide metabolism	Purine metabolism	104			Down	Down/Mixed
		Pyrimidine metabolism	73	Down	Down/Mixed	Down	Down/Mixed
	Lipid metabolism	Fatty acid degradation	24	Up	Up		
Organismal Systems	Circulatory system	Cardiac muscle contraction	41	Up	Up		
	Digestive system	Carbohydrate digestion and absorption	15	Up	Up		
		Fat digestion and absorption	19	Up	Up/Mixed	Up	Up

		Gastric acid secretion	27	2D	Up		
		Mineral absorption	25	Up	Up		
		Protein digestion and absorption	40	Up	Up		
		Vitamin digestion and absorption	17	Up	Up		
	Endocrine system	Adipocytokine signaling pathway	35	Up/2D	Up/Mixed		
		Insulin secretion	37	2D	Up		
		PPAR signaling pathway	42	Up	Up/Mixed		
	Excretory system	Proximal tubule bicarbonate reclamation	11	Up	Up		
	Immune system	B cell receptor signaling pathway	39			2D	Mixed
		Fc epsilon RI signaling pathway	29			2D	Down
		Natural killer cell mediated cytotoxicity	40	2D	Down		

Table 5.3 Pathways found to be differentially expressed between control and stress conditions in reciprocal hybrids by both gage and romer packages. The direction of change shown describes the expression of the pathway under stress condition relative to control condition. The terms “2D” and “Mixed” are used to describe pathways in which genes showed bidirectional change. “Genes” refers to the number of genes included in the gene set test.

KEGG group	KEGG subgroup	Pathway	Genes	W♀D♂		D♀W♂	
				gage	romer	gage	romer
Cellular Processes	Cell growth and death	Cell cycle	88	Down	Down/Mixed	Down	Down/Mixed
		Cell cycle – yeast	54	Down	Down/Mixed	Down	Down/Mixed
		Meiosis – yeast	41	Down	Down/Mixed	Down	Down/Mixed
Environmental Information Processing	Signal transduction	ErbB signaling pathway	40			2D	Down
		HIF-1 signaling pathway	47			Up	Up
		MAPK signaling pathway	110			2D	Up
	Signaling molecules and interaction	Neuroactive ligand-receptor interaction	112	2D	Up	Up/2D	Up
Genetic Information Processing	Folding, sorting and degradation	Proteasome	40	Down	Down/Mixed	Down	Down/Mixed
	Replication and repair	Base excision repair	28	Down	Down/Mixed	Down	Down/Mixed
		DNA replication	33	Down/2D	Down/Mixed	Down/2D	Down/Mixed
		Homologous recombination	20	Down	Down/Mixed	Down	Down/Mixed
		Mismatch repair	18	Down	Down/Mixed	Down/2D	Down/Mixed
		Nucleotide excision repair	35	Down	Down/Mixed	Down	Down/Mixed
	Transcription	Spliceosome	109	Down	Down/Mixed	Down	Down/Mixed
	Translation	Ribosome biogenesis in eukaryotes	64	Down	Down/Mixed	Down	Down/Mixed

		RNA transport	111	Down	Down		
Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle)	22	Up	Up	Up	Up
		Galactose metabolism	16	Up	Up/Mixed		
		Glycolysis / Gluconeogenesis	30	Up	Up/Mixed	Up	Up/Mixed
		Starch and sucrose metabolism	21			Up	Up
	Energy metabolism	Carbon fixation in photosynthetic organisms	15			Up	Up/Mixed
		Oxidative phosphorylation	105	Up	Up	Up	Up
	Glycan biosynthesis and metabolism	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	11	2D	Up		
	Lipid metabolism	Fatty acid degradation	24	Up	Up	Up	Up
		Glycerolipid metabolism	25	Up	Up		
		Glycerophospholipid metabolism	44			Up	Up
	Metabolism of cofactors and vitamins	One carbon pool by folate	13			Down	Down/Mixed
	Nucleotide metabolism	Purine metabolism	104	Down	Down/Mixed	Down	Down/Mixed
		Pyrimidine metabolism	73	Down	Down/Mixed	Down	Down/Mixed
Organismal Systems	Circulatory system	Cardiac muscle contraction	41	Up	Up/Mixed		
		Vascular smooth muscle contraction	55	2D	Up	Up/2D	Up
	Digestive system	Carbohydrate digestion and absorption	15	2D	Up		
		Fat digestion and absorption	19	Up	Up	Up	Up
		Gastric acid secretion	27			Up/2D	Up
		Pancreatic secretion	43			Up	Up
		Protein digestion and absorption	40			Up	Up
		Vitamin digestion and absorption	17			Up	Up
	Endocrine system	Adipocytokine signaling pathway	35	2D	Up	Up/2D	Up
		Insulin secretion	37			Up/2D	Up
		Insulin signaling pathway	56			Up	Up
		PPAR signaling pathway	42	Up	Up/Mixed	Up	Up/Mixed
	Environmental adaptation	Circadian rhythm	19			2D	Up
	Immune system	T cell receptor signaling pathway	61			2D	Down
	Nervous system	GABAergic synapse	38			Up	Up
		Glutamatergic synapse	50			2D	Up
		Long-term potentiation	28			2D	Up
		Retrograde endocannabinoid signaling	46			2D	Up

5.3.3 Heritability

The use of reciprocal hybrids allowed exploration of gene expression heritability. Additivity (38%-46%) accounted for most differential expression patterns detected among the four crosses, followed by maternal dominance (18%-32%) (Figure 5.3, Table 5.4). On average 42% of the differentially expressed genes exhibited intermediate hybrid expression relative to the pure crosses. However, there was a greater difference in the relevance of additivity between the stressed reciprocal hybrids (38% and 46%), than between controls (43% and 41%). The same was true for maternal dominance, with the percentages of differentially expressed genes in the reciprocal hybrids exhibiting this inheritance pattern under the control treatment being consistent (26% and 24%), whereas there was a greater difference between the hybrids under stress (32% and 18%).For most comparisons, maternal dominance was more than double that of paternal dominance, however, in case of the stressed D♀W♂ hybrids, the difference was considerably smaller; paternal and maternal dominance accounting for 15% and 18% of the differentially expressed genes respectively. There were more pronounced maternal effects detected in the W♀D♂ than in the D♀W♂ hybrids at the expense of additivity under stress conditions, suggesting that the genes responsible for the imbalance are specifically wild maternal and not just maternal dominant. Genes that were wild dominant in the W♀D♂ hybrids, and were additive/wild dominant in the D♀W♂ hybrids under stress were considerably more abundant than genes that were additive/domesticated dominant in the W♀D♂ hybrids, but were domesticated dominant in the DW hybrids under stress (34 vs 9 genes). Only seven of these genes were differentially expressed under control conditions, under which state four of them showed maternal dominance (see Appendix for details). The expression of the nominal wild dominant genes was more consistent in the domesticated crosses than in the wild crosses under stress (Figure 5.4). The products of many of the genes found to be wild (over)dominant in the W♀D♂ hybrids, and additive/wild dominant in the D♀W♂ hybrids under stress conditions, were enzymes involved in metabolism, in particular lipid and energy.

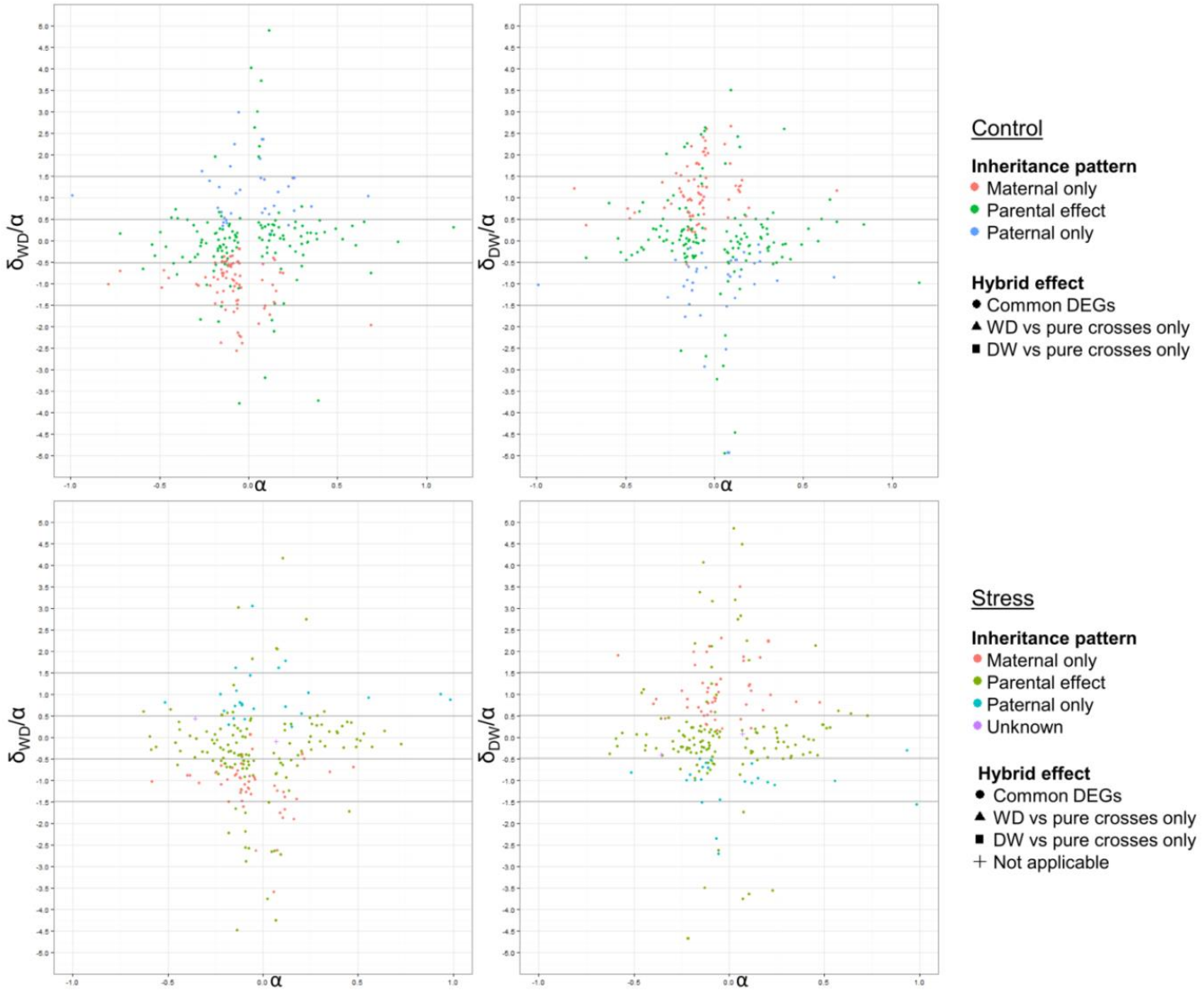
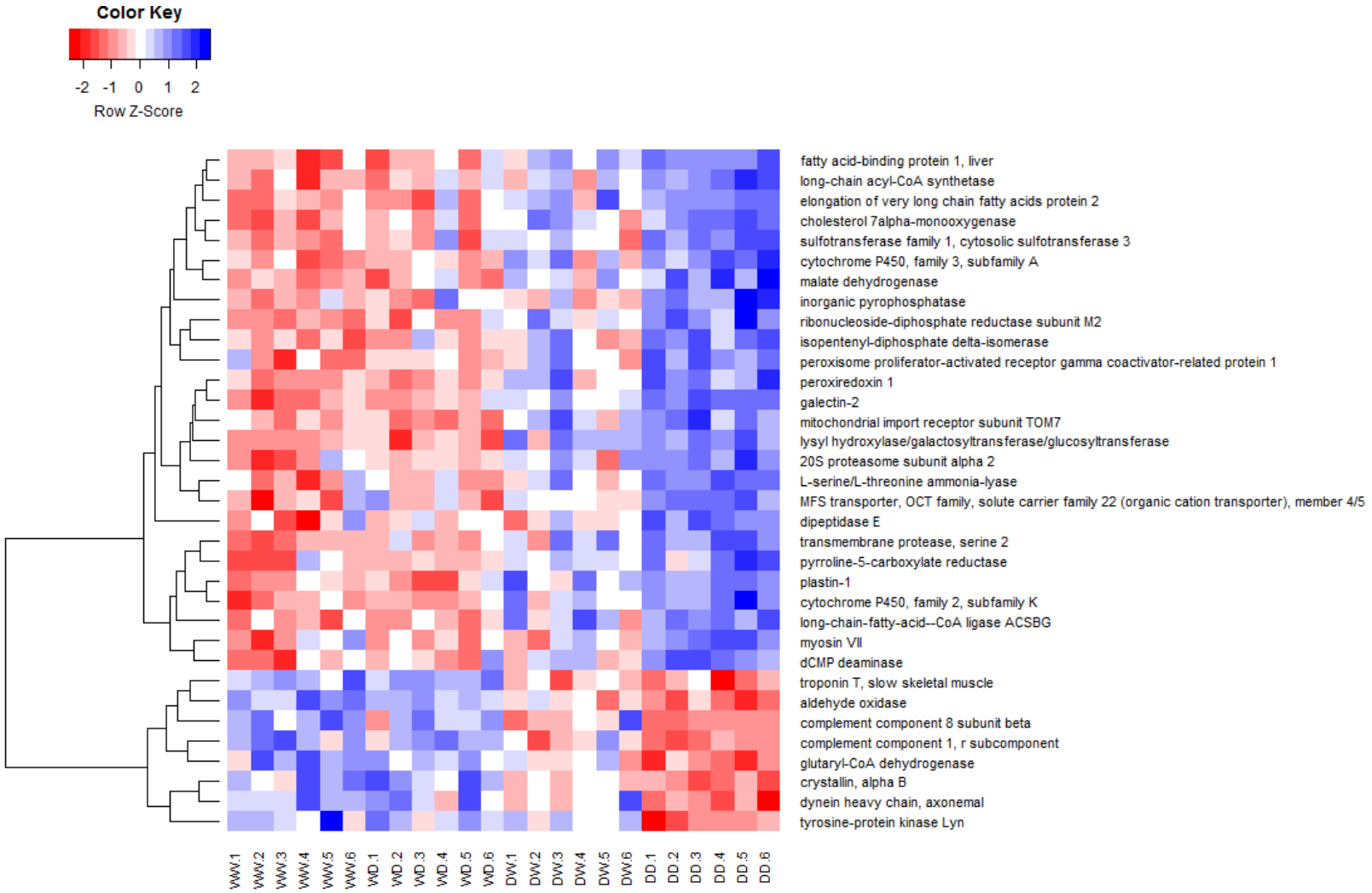


Figure 5.3 Visual representation of heritability of genes differentially expressed between crosses in control (graphs on top) and stress (graphs on bottom) states. Heritability was plotted for both reciprocal hybrids; W♀ x D♂ (on the left) and D♀ x W♂ (on the right). $\alpha > 0$ / $\alpha < 0$ is characteristics to genes that are down/up regulated in domesticated compared to wild fish and $-0.5 < \delta/\alpha < 0.5$ corresponds to additivity, $-1.5 < \delta/\alpha < -0.5$ to wild dominance, $0.5 < \delta/\alpha < 1.5$ to domesticated dominance, and if δ/α falls out of the interval −1.5-1.5, then over-dominance of the expression of the transcripts studied.

Table 5.4 Proportions of the differentially expressed genes displaying various inheritance patterns in the reciprocal hybrids relative to the expression of pure crosses under control and stress conditions.

Hybrid type / Heritability pattern	Control		Stress	
	W♀ x D♂	**D♀ x W♂**	**W♀ x D♂**	**D♀ x W♂**
Wild over-dominant	10.7%	8.9%	12.6%	7.6%
Wild dominant	25.8%	11.2%	31.6%	14.8%
Additive	42.9%	40.5%	37.9%	46.2%
Domesticated dominant	11.9%	23.6%	11.2%	17.6%
Domesticated over-dominant	8.7%	15.8%	6.8%	13.8%
Number of unique genes	252	259	206	210

Figure 5.4 Hierarchical clustering of the normalised expression values of the genes that were identified as wild (over)dominant in the W♀ x D♂ hybrids, and additive/wild dominant in the D♀ x W♂ hybrids under stress conditions.



5.4 Discussion

Atlantic salmon have been selectively bred since the early 1970s and as a result wild and farmed Atlantic salmon populations have genetically diverged (Gross, 1998) providing a good model in which to study the genetic effects of trait-specific directional selection, as well as domestication in general. Evolutionary change can be reflected in gene expression differences (Carroll, 2005; King and Wilson, 1975). In this respect, transcriptional differences between wild and domesticated Atlantic salmon stocks have been previously recognised and studied in whole animals (Bicskei et al., 2014; Roberge et al., 2008, 2006). Variation in stress responsiveness between genotypes has previously been reported for commercial Atlantic salmon breeding programs (Kittilsen et al., 2009). Considering that wild and domesticated fish are adapted to different environments, some aspects of the stress response might be expected to differ. Hatchery rearing alone (Naslund et al., 2013), as well as longer term domestication have been previously shown to reduce stress responsiveness of Atlantic salmon (Solberg et al., 2013a). In the current study transcriptional divergence between stocks and in response to stress was supported by multiple lines of evidence. Separation of stress and control, as well as wild, hybrid and domesticated samples was clearly evident from the PCA analysis. Statistical analysis identified a large number of differentially expressed transcripts in response to stress and between the stocks. Moreover, functional analyses found numerous functions that were differentially perturbed between the crosses and/or in response to stress. However, using whole individuals meant that tissue specificity of gene expression was lost and it needs to be considered during biological interpretation of the differences. In addition, although organisms respond to stress via coordinated changes of their gene expression, it may be achieved through post-transcriptional control (Holcik and Sonenberg, 2005; Yamasaki and Anderson, 2008). These changes are likely to be undetected when comparing mRNA abundance.

5.4.1 Effects of domestication on stress response

Domestication results from a combination of selection processes. Traits for desired characteristics are methodically selected for, while additional traits may be inadvertently co-selected. Individuals that respond best to the selection pressures and are most adapted to their environment are promoted as broodstock. Changes in baseline responses to anthropogenic stimuli have been suggested to be an important aspect of domestication (Price, 2002, 1984). Increased stress resilience is one of the traits suggested to differentiate wild and domesticated Atlantic salmon (Gross, 1998). Although differential stress responsiveness might therefore be expected as a signature of domestication, this was not apparent from the ANOVA analyses, which showed no statistically significant interaction between stock and stress response in either analysis (+/- hybrid data). Functional analysis, however, suggested that gene expression in some pathways may reflect a stock-specific stress response. Inclusion of hybrid data in ANOVA analyses of transcript expression for fish under stress / control conditions increased the number of differentially expressed transcripts detected, which could be indicative of heightened responsiveness to stress in hybrids.

5.4.1.1 Common responses to stress in wild and domesticated origin fish

Cells respond to stress by reprogramming their metabolism and shifting energy generated by anabolic processes to the repair of stress-induced molecular damage via alteration of the protein translation machinery. In particular, mRNA translation initiation shifts focus from ‘housekeeping’ to repair processes (Yamasaki and Anderson, 2008). Overall, stress is thought to reduce global translation throughout the organism in order to preserve cellular energy (Holcik and Sonenberg, 2005). This was reflected in the current study with down-regulation of *genetic information processing* in response to stress being detected, including pathways of *replication and repair*, *transcription* and *translation*. *Cell cycle* and *meiosis* pathways, related to *cell growth and death*, were similarly affected. In addition, vertebrate stress response involves increased oxygen uptake and transfer, mobilization of energy substrates and reallocation of energy away from growth and reproduction and towards restoration of homeostasis. Increased

metabolic rate, as indicated by positive stress-correlated plasma glucose or oxygen consumption, is also associated with the stress response as is immunosuppression (Wendelaar Bonga, 1997). Data from this study indicated that stress increased metabolic processes, including *carbohydrate, lipid*, and *protein metabolism* and activities involving *co-factors and vitamins*. Up-regulation of *energy metabolism*, *circulatory*, *digestive* and *endocrine systems* and down regulation of *immune pathways* were also characteristic for all stressed fish.

5.4.1.2 Stock specific stress response

In addition to functional differences shared across stocks in response to stress, the data was also indicative of stock specific stress response. Although, functional differences were found between wild and domesticated pure crosses, as well as between the hybrid stocks, these were often mild, only supported by one or the other gene set enrichment method and as such should be investigated further.

In contrast with ANOVA analysis, functional analyses of responses to stress identified apparent differences between wild and domesticated origin fish for a number of biological functions, In particular, stress only seemed to affect *signaling molecules and interaction* pathways, *cytokine-cytokine and neuroactive ligand-receptor interactions* in domesticated fish, whereas *metabolic pathways*; *glycolysis/gluconeogenesis* and *Fatty acid degradation*, and the majority of *digestive and endocrine system* pathways seemed to be characteristic of wild stress response. The expression of all of the unique differences was enhanced in the stressed compared to control fish. Although many of these were marginal, being identified by only one or other of the two analytical tools employed (gage or romer), the stress-associated up-regulation of *mineral absorption* and *protein digestion and absorption* pathways in wild origin fish *cf*. domesticated origin fish was fully supported by both packages.

Inclusion of the reciprocal hybrids contributed to an approximately 67% increase of the differentially expressed transcripts detected in response to stress. In addition, there were more pathways differentially expressed in response to stress in reciprocal hybrids

than in pure crosses. This suggests that the stress response of the reciprocal hybrids was more substantial and/or more variable, than that of the pure crosses. It has been proposed that radical genetic changes, such as genes entering from one population to another, may disrupt adaptation and put homeostatic balance at risk (Rauw, 2009). In hybrid fish, disruption of adaptation may therefore have engendered a need for more extensive responses to stress in order to maintain homeostatic balance. Enriched pathways observed in both hybrids included *signal transduction* and *nervous system*, which were also highlighted in previous studies of fish of wild and farmed origins (Bicskei et al., 2015, 2014). Members of these enriched pathways included *MAPK signaling*, *glutamatergic synapse*, *long-term potentiation* and *retrograde endocannabinoid signaling* all of which are known to be affected by stress and have been implicated in food intake regulation/growth and/or domestication. MAPK is involved in stress response, growth (Morrison, 2012) and domestication (Amaral et al., 2011; Bicskei et al., 2015; Nätt et al., 2012; Park et al., 2014; Yang et al., 2014), *glutamatergic synapse* has been implicated in stress response, feed intake regulation and domestication (Bicskei et al., 2015; Li et al., 2014; Moon et al., 2015), *long-term potentiation* has been associated with learning, memory consolidation (Wei et al., 2012) and domestication (Bicskei et al., 2015; Li et al., 2014; Nätt et al., 2012). *Retrograde endocannabinoid signaling* is affected by stress (Castillo et al., 2012) and regulates feeding behaviour (Elphick, 2012).

Hybrid type varied in some aspects of their response to stress. Overall, there were more differentially expressed pathways detected in D♀W♂ hybrids, than in W♀D♂ hybrids, primarily affecting functional groups of *signal transduction*, *digestive, endocrine* and *nervous system* pathways that were mainly up regulated in response to stress. Out of these functions, *protein digestion and absorption, HIF-1 signalling* and *GABAergic synapse* pathways were consistently present in response to stress in D♀W♂ hybrids but absent in W♀D♂ hybrids. *HIF-1* is a transcription factor that functions as the master regulator of oxygen homeostasis and which is induced in

response to reduced oxygen availability and/or by other stimulants, including nitric oxide and various growth factors (Zagórska and Dulak, 2004). GABA is considered as one of the most abundant neurotransmitters in the vertebrate central nervous system. It is involved in a number of neuroendocrine processes including the modulation of feeding and stress response, as well as the stimulation of neural development and differentiation and reproduction (Martyniuk et al., 2005).

Some of the stress responsive functional differences that differed between the pure stocks and reciprocal stocks were shared. For example, a larger number of digestive and endocrine systems related pathways were perturbed in response to stress in the wild, than in the domesticated pure stock. The same trend, affecting the same pathways was observed in the D♀W♂ hybrids compared to W♀D♂ hybrids. Although largely the result of either gage or romer failing to detected some of these pathways, it indicates that for digestive and endocrine functions, wild pure and D♀W♂ hybrids had a more consistent and/or stronger stress response, than pure domesticated and W♀D♂ hybrid stocks.

5.4.2 Effects of domestication on other traits

Aquaculture and natural environments differ across a broad range of parameters over and above stress this being reflected in differential selection (Werf et al., 2009). Breeding in domesticated fish is controlled via selection programs commonly targeting economically important traits, such as increased growth rate and body size, late maturation, disease resistance and flesh quality. Although high performance in respect to these traits increases the chances of an individual domesticated fish being selected for broodstock, such traits are less likely to be similarly advantageous under natural conditions (Skaala et al., 2006). At the same time, domesticated animals are provided with a controlled environment, where certain natural selection pressures may be relaxed. For example, since predators are absent and food is abundant, predator avoidance and competition for food have reduced adaptive significance in captivity (Mignon-Grasteau et al., 2005; Price, 1984), but see (Skaala et al., 2013; Solberg et

al., 2015). Possibly for this reason, survival of farmed salmon offspring is inferior to that of the offspring of wild salmon in the wild (Besnier et al., 2015; Fleming et al., 2000; McGinnity et al., 2003; Skaala et al., 2012).

5.4.2.1 Biological functions down-regulated in fish of domesticated origin

Cellular signalling functions in homeostasis by controlling cell replication, differentiation and apoptosis and helps to regulate metabolic events. Stimuli for responses include nutritional state, inflammatory signals or alteration of the organism's physical environment (Elliott and Elliott, 2009), these being factors likely to differ between natural and artificial niches. Down-regulation of *signalling* pathways in domesticated fish may be indicative of these animals being better adapted to the more consistent farm environment such that they require less sensitivity / capacity to maintain homeostasis.

Reduction of information acquisition and processing systems, including those involving sensory organs and synapses with transmitter substances for information processing, has been proposed to be a consequence of domestication (Hemmer, 1990). The current study supports this hypothesis, with both *cell communication* and *nervous system* pathways being found to be down-regulated in fish of domesticated origin compared to wild. Further support comes from previous studies, where for the same stocks, *cell communication* pathways *gap junction* and *focal adhesion* were observed to be differentially expressed between wild and domesticated origin embryos (Bicskei et al., 2015) and *nervous system* related pathways *synaptic vesicle cycle* and *serotonergic synapse* were down regulated in the domesticated origin sac fry (Bicskei et al., 2014). *Glutamatergic synapse* was also identified as differentially perturbed/down regulated in domesticated embryo/sac fry respectively (Bicskei et al., 2015, 2014). Generally, decreased serotonergic activity is associated with dominance, boldness and aggression (Lillesaar, 2011); behaviours more prominent in domesticated fish when compared to wild counterparts in the hatchery environment (Fleming and Einum, 1997). Glutamate is a major excitatory neurotransmitter that

regulates various behaviours and emotions and is involved in learning and memory. Changes in glutamate metabolism are suggested to have occurred during domestication of dogs (Li et al., 2014) and pigs (*Sus scrofa domesticus*) (Moon et al., 2015). Expression of glutamate receptors seems to affect the neural control of eating behaviours in pigs (Moon et al., 2015), with their deficiency having been shown to decrease fear and anxiety in mammals and their up regulation having been hypothesised to enhance excitatory synaptic plasticity in dogs (Li et al., 2014). Up regulation of glutamate activity and hence increased fear and anxiety in dogs compared to wolves is contrary to what one might expect in response to domestication. However, the authors argued that its beneficial effects in terms of strengthening the dogs' learning and memory abilities outweighed the effects of fearfulness since it aids the accurate interpretation of human behaviour.

Another major down-regulated functional group detected in domesticated fish in the current study, in domesticated embryos (Bicskei et al., 2015) and in sac and feeding fry (Bicskei et al., 2014) belonging to the same stocks was *immune system*. In fish, the neuroendocrine and immune systems are interlinked through shared cytokines and neuropeptides (Nardocci et al., 2014; Tort, 2011) and most of the differentially expressed immune pathways identified in the current study were involved in signalling. Domestication involves adaptation to a human-controlled environment. Since the importance that particular traits have in the wild, shifts during selection for domestication, the energy invested in them similarly has to be optimised to the new environment. In part this must be achieved through the (re)allocation of resources, and such a trade-off has been identified between growth and immune function, especially in livestock selected for increased production traits (Rauw, 2012). In line with the resource allocation theory, data from the current study showed down-regulation of *immune pathways* in domesticated fish and simultaneous up-regulation of *metabolism*, *endocrine* and *digestive systems* and *genetic information processing*. This is consistent with previous studies that have demonstrated significantly increased growth

rates in farmed salmon in comparison with their wild counterparts under identical conditions(Glover et al., 2009; K. A. Glover et al., 2006; Solberg et al., 2013a, 2013b)

5.4.2.2 Biological functions up-regulated in fish of domesticated origin

Greater consumption and more efficient utilization of fish feed for growth was reported for Atlantic salmon selected for increased growth over five generations compared to wild counterparts (Thodesen et al., 1999). In addition, selection for growth was suggested to be likely to result in individuals with more active endocrine systems (Fleming et al., 2002). Such differences were also evident from the results of the current study, with up-regulation of *metabolism* and in particular of *carbohydrate* and *lipid metabolism* and *digestive* and *endocrine system* pathways in the domesticated compared to wild fish. In addition, *cellular processes*, such as *cell cycle* and *peroxisome* and *genetic information processing*, including *DNA replication*, *mRNA transcription and translation,* indicative of protein production and growth, were also more highly represented in fish of domesticated origin than in wild origin counterparts.

Functional groupings and regulation of the differentially expressed transcripts detected between fish of domesticated and wild origins were largely consistent between control and stress conditions, as shown by the biological pathways identified and their direction of change. Overall, fewer pathways were identified as differentially expressed in the stress state. This could be a result of individual differences in stress response that may have introduced greater variability in the data and thereby reduced the ability to detect consistent differences in transcript expression; however, the adoption of a pooled design in the current study should decrease the effects of individual variation. Differences were observed in *digestive system*; including *protein and vitamin digestion and absorption* and *mineral absorption* pathways. As these pathways were up-regulated in domesticated compared to fish of wild origin and were up regulated in response to stress only in the wild fish, it is likely that under stress conditions the increased wild expression reduced the difference between wild and domesticated fish of these pathways to a non-significant level.

5.4.3 Heritability of transcriptomic differences

Most transcriptomic differences detected between the cross types reflected additive behaviour, with c. 40% of differentially expressed transcripts exhibiting intermediate expression in hybrids compared to the pure crosses. Additive genetic variation has been suggested to be characteristic of important Atlantic salmon traits, such as fitness, survival (Ferguson et al., 2007; Dylan J Fraser et al., 2010), growth and behaviour (Dylan J Fraser et al., 2010; Glover et al., 2009; Solberg et al., 2013a). Moreover, additive inheritance of gene expression is widespread between conspecifics from widely divergent salmonid populations, including wild and domesticated Atlantic salmon (Bicskei et al., 2015, 2014; Debes et al., 2012), brook charr (Bougas et al., 2010) and dwarf and normal lake white fish (Renaut et al., 2009).

Parental effects were differentiated from the effects of domestication by investigating the heritability patterns of the reciprocal hybrids. The majority of the genes showing dominance (18-32%) followed the behaviour of the dam in hybrids and therefore it is clear that that dominance was largely a maternal property, being irrespective of stock origin. Fewer genes displayed paternal dominance behaviour (11-15%), an observation also reported for wild and domesticated brook charr, where 40% of the differentially expressed genes exhibited maternal and 5% paternal dominance (Bougas et al., 2010). Maternal effects are common in salmonids and have been mainly associated with egg and nest quality (Green, 2008), with egg and alevin size and survival similarly reported to be maternally influenced (Einum and Fleming, 2000, 1999; Houde et al., 2011; Skaala et al., 2012; Solberg et al., 2014). Maternal effects are likely to be influenced by both genetic and environmental sources of variation (Wolf and Wade, 2009). The influence of these components on the phenotype are subject to change over time, and a shift from larger maternal environmental effects to larger genetic effects has been shown during the development of Atlantic salmon (Houde et al., 2015). Maternal influence tends to decline over time, including that due to transcriptomic differences (Bougas et al., 2013a). This trend was evident for the extent of maternal over-dominance, for the same stocks studied here. The number of

transcripts governed by over-dominance steadily decreased from approximately 20% in the embryo stage (Bicskei et al., 2015), through a mean of 13% to 5% in fry approximately 3 weeks (Table 5.4) and 5 weeks (Bicskei et al., 2014) post first feeding respectively.

The contribution from additivity and maternal dominance, was consistent between reciprocal hybrids of the control state, but less so in the stress state. This was due to the relatively large proportion of genes that were wild dominant in the W♀ x D♂ hybrids, and were additive/wild dominant in D♀ x W♂ hybrids under stress. This suggests that these genes were under wild dominance, as opposed to maternal dominance regardless of the maternal status. Maternal effects can be adaptive or maladaptive depending on whether the maternal environment is reflective of the offspring's environment. There are a range of factors known to influence environmental maternal effects including maternal diet and stress experiences (Green, 2008) that likely vary between natural and farm conditions. Since many of the genes indicative of maternal environmental effects are stress responsive and are involved in lipid and energy metabolism, their expression pattern could be affected by differences in the way wild and domesticated fish metabolise feed, experience stress and produce energy in response to it. In the current study the expression of the affected genes was more consistent in domesticated origin fish than it was in wild origin fish under stress conditions. This may reflect greater variability of expression of these genes in response to stress in the wild population. Reduced genetic variation has been previously reported for fitness related QTLs in response to domestication, possibly due to genetic sweeps (Besnier et al., 2015).

5.5 Conclusions

This study investigated the functional significance and heritability of transcriptomic differences between fry stage offspring of Atlantic salmon of wild and domesticated origin, maintained under standard hatchery and acute stress conditions. Differences observed are discussed in terms of the contrasting selection pressures acting on

natural and aquaculture populations. Although a higher number of responsive pathways were detected in wild origin fish in response to stress, many of the affected pathways were common to both stocks. The major stress-responsive functional groups were indicative of mobilisation and re-allocation of energy. Reciprocal hybrids exhibited similar transcriptomic stress responses to pure domesticated and wild origin stocks, however, some functions that were detected to be differentially expressed between wild and domesticated fish were also found between stress and control hybrids. Additivity and maternal dominance were observed to be the most important modes of inheritance for differential transcript expression detected between the stocks. Our transcriptomics results indicate the maladaptation of domesticated fish to natural conditions and highlight the relevance of additivity and maternal dominance. This combined with the principal route of gene flow involving domesticated females mating with native males (Fleming et al., 2000) suggests that the hybrid offspring of escapees will also be heavily affected by the likely negative impact of domestication.

Chapter 6 - A comparative analysis of the transcriptomes of wild and domesticated Atlantic salmon (*Salmo salar* L.) embryo, sac and feeding fry

6.1 Background

Atlantic salmon has been selectively bred for over 10 generations, targeting production related traits and leading to the genetic divergence of domesticated fish from its wild counterparts (Glover et al., 2009). However, the traits considered preferable under farm conditions are unlikely to be advantageous under natural conditions. Indeed, domesticated fish have been found to demonstrate initial faster growth but poorer survival rate and lower productivity in natural environment, when compared to wild fish (McGinnity et al., 2003; Reed et al., 2015; Skaala et al., 2012). With the rapid expansion of the Atlantic salmon industry, the potential impact of farm escapees into natural populations remains a concern (Taranger et al., 2015).

In previous chapters (3-5) experiments were performed in an attempt to elucidate the transcriptomic differences between wild and domesticated Atlantic salmon. These focused on the characteristics of different early life stage specific transcriptomic differences between domesticated and wild Atlantic salmon. Gene expression is plastic and subject to temporal change throughout life. For example, in the first 90 days of the life of the Atlantic salmon, differential expressions among at least several thousand genes can be detected (Jantzen et al., 2011). Hence, comparing the transcriptome of wild and domesticated salmon at various life stages is likely to reveal different genes and biological pathways affected by domestication. The analysis presented in this chapter aims to compare these differences in an attempt to identify general trends that may apply to Atlantic salmon domestication regardless of the life stages. In order to keep the comparisons consistent, data from the experiments were re-analysed from the statistical analysis phase onwards, using the same parameters, software and

annotations. Moreover, the functional analysis was expanded by employing multiple methods for a more thorough exploration of the data.

Previous studies exploring transcriptional differences resulting from domestication in Atlantic salmon have been reported (Roberge et al., 2008, 2006). Their work also focused on RNA extracted from whole fish early in its life history. Presumably, though not overtly stated, this approach was taken to avoid the developmental effects of the early growth divergence of wild and domesticated fish. However, while Roberge and colleagues sampled at initial swim-up phase, samplings undertaken throughout the current studies were specifically timed to avoid transitional event life stages, when major changes in gene expression may occur due to entering a different developmental / physiological state. A major limitation of transcriptomics experiments conducted on whole individuals is the loss of information relating to tissue specific gene expression. However, rapid sampling, essential to avoid unintentional transcriptomic alterations, and difficulty in consistently dissecting organs from early life stages precluded tissue expression investigation. An important aspect of the work by Roberge and colleagues was the use of two wild and domesticated stocks (one of each of Norwegian and Canadian origin) that provided the opportunity for the detection of parallel transcriptomic changes in response to domestication. They found that parallel changes among stocks were rare at gene level, and mainly occurred in similar biological functions. A unique feature of the current work is that multiple early life stages were investigated, and analysed reciprocal hybrids separately allowing for the elucidation of modes of heritability governing the expression of the transcriptomic differences identified between the crosses.

6.2 Methods

6.2.1 Data collection

Previously published data was used from three separate microarray studies conducted on four different ages of Atlantic salmon. A summary of the microarray interrogations are provided in Table 6.1. For more detailed descriptions see Chapters 3-5 were

individual experiments are thoroughly discussed. For each experiment, pure wild, pure domesticated and hybrid crosses were created using the wild Figgjo (hereafter W for wild) and domesticated Mowi (hereafter D for domesticated) stocks. Crosses are consistently defined as Dam x Sire.

Table 6.1 Details of the experiments compared.

	Embryo	Sac fry	Fed fry 3 weeks	Fed fry 5 weeks
Chapter	4	3	5	3
Crosses	D×D, D×W, W×D, W×W	D×D, D×W, W×W	D×D, D×W, W×D, W×W	D×D, D×W, W×W
Life stage	Eyed egg (10 weeks post fertilization)	Sac fry (7 weeks post hatch / 1 week before swim up)	3 weeks following first feeding	5 weeks following first feeding
Sampled	410°d post-fertilization	256°d post-hatch	985°d post-fertilization	867°d post-hatch
Families	Same as fed fry 3 weeks	Randomly sampled from 10 families	Same as embryo	Randomly sampled from 10 families

6.2.2 Identification of differentially expressed transcripts and genes

To identify differentially expressed transcripts Welsh T-tests (unpaired unequal variance) with 10% FDR (Benjamini-Hochberg) were performed within each experiment for the domesticated vs wild contrast in GeneSpring version 13.0 (Figure 6.1). Differentially expressed transcripts that were present in at least three comparisons were KEGG annotated, or if not available BLASTx annotated, and hierarchical clustered using the *heatmap.2* function of the *gplots* package (Warnes et al., 2014) from the *R software v.3.1.3* (R Core Team, 2015)(Figure 6.2).

6.2.3 Functional analysis

All probes that passed quality filtering, as defined by Chapters 3-5, were considered for the functional analysis. The two microarray designs (salar2 and salar3) used across experiments only differed in 266 Atlantic salmon experimental probes, i.e. 264 duplicate probes present salar2 that were present as singletons in salar3, and two sex

specific probes that were added to salar3. Thus the data were deemed to be comparable. KEGG based functional annotation was obtained and only unique genes were considered for downstream analysis. Where multiple probes were associated with the same KO number, probes that had the lowest p-value based on T-tests (unequal variance) for the contrast of domesticated and wild pure crosses were chosen. Functional analysis was performed using three packages from *R software v.3.1.3* (R Core Team, 2015) in order to achieve more robust results, i.e. those supported by different methods. The three analyses were rank based *GAGE* analysis (Generally Applicable Gene-set/Pathway Analysis) (Luo et al., 2009) implementing Mann Whitney U tests and *romer* and *roast* functions from the *limma package* (Linear Models for Microarray Data) (Smyth, 2004). For *gage* and *roast* results a corrected *p-value* for significance of <0.1 was applied, whereas for *romer* the cut-off for the number of genes was 10 with a *p-value* < 0.05. Both uni (Figure 6.3) and bidirectionally (Figure 6.4) perturbed pathways were considered. The former consists of genes that were either up or down regulated, but not both within the same pathway (1d), whereas the latter has genes that are both up and down regulated within the same pathway (2d/mixed).

6.2.4 Heritability

Heritability analysis of the differentially expressed genes identified by 1-way ANOVA (unequal variance) was performed with 10% FDR (Benjamini-Hochberg) and Student Newman-Keuls (SNK) *post-hoc* analysis was applied using GeneSpring software. To avoid repeated counting of the same gene, only transcripts that had KEGG annotation available were chosen and where multiple probes were present for the same gene, the probe with the highest overall significance (lowest geometric mean calculated on the p-values across life stages) was chosen. Where functional groups are presented, genes that were present in any of the pathways of the five main KEGG functions (*organismal systems, metabolism, genetic information processing, environmental information processing, and cellular processes*) are shown. Although these genes are unique to the sub-functional groups (e.g.: *immune system*), they may be present in multiple sub

functions and therefore may appear multiple times within the main KEGG function (e.g.: *organismal systems*)

Where genes were assigned to heritability categories based on the comparison in which they were significantly differentially expressed, it was performed as follows:

Maternal effect: differential expression between

W♀ x W♂vs D♀ x W♂ or D♀ x D♂vs W♀ x D♂

Paternal effect: differential expression between

W♀ x W♂ vs W♀ x D♂ or D♀ x D♂ vs D♀ x W♂

Parental effect: influenced by both maternal paternal effects

Maternal only: unique to maternal effect

Paternal only: unique to paternal effect

In addition additivity; α = (wild-domesticated)/2 and dominance parameters; δ = (wild + domesticated)/2-hybrid were calculated from normalised intensity values and α and δ/α were plotted using the ggplot2 package (Wickham, 2009). Considering that, by definition, a transcript whose expression value in hybrids corresponds to the mid-value of the parents' is additive (i.e.: perfect additivity: δ/α = 0) and that a transcript whose hybrid gene expression value resembles more closely one parent or another is dominant (i.e.: domesticated dominance: δ/α = 1 and wild dominance: δ/α = -1), by halving the intervals we can presume that transcript expression corresponds to:

- additivity if $-0.5 < \delta/\alpha < 0.5$
- wild dominance if $-1.5 < \delta/\alpha < -0.5$
- domesticated dominance if $0.5 < \delta/\alpha < 1.5$
- Over-dominance if δ/α falls out of the interval −1.5-1.5.

Moreover, according to the formula, the direction of the additive parameter is indicative of the gene expression regulation between wild and domesticated pure crosses:

- α >0 is characteristics to genes that are down regulated in domesticated compared wild fish
- α <0 is characteristics to genes that are up regulated in domesticated compared wild fish

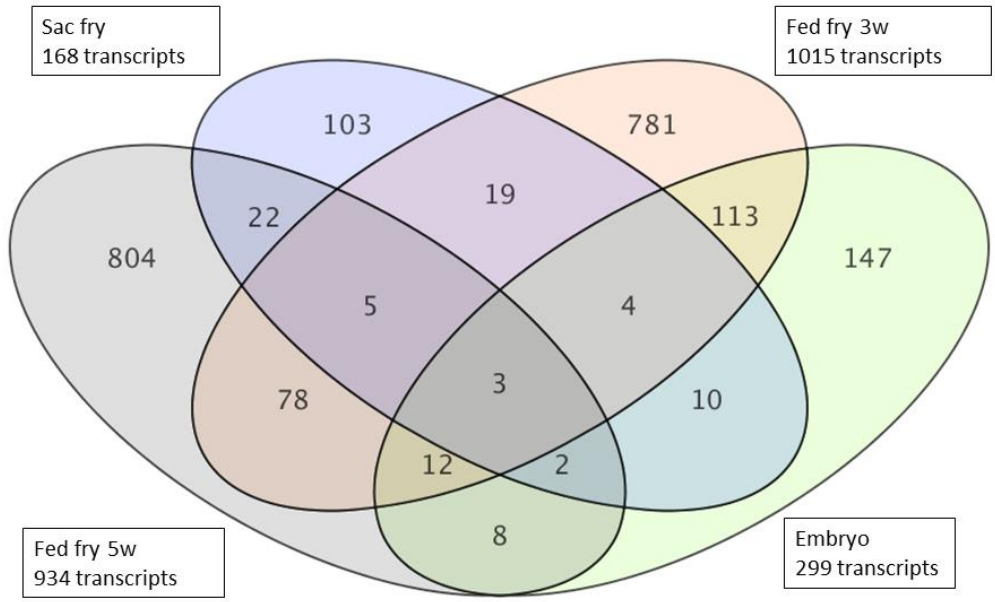
For ease of interpretation, genes with |δ/α |≥ 2 were plotted as 2 on the scatter graph.

6.3 Results

6.3.1 Differentially expressed transcripts and genes

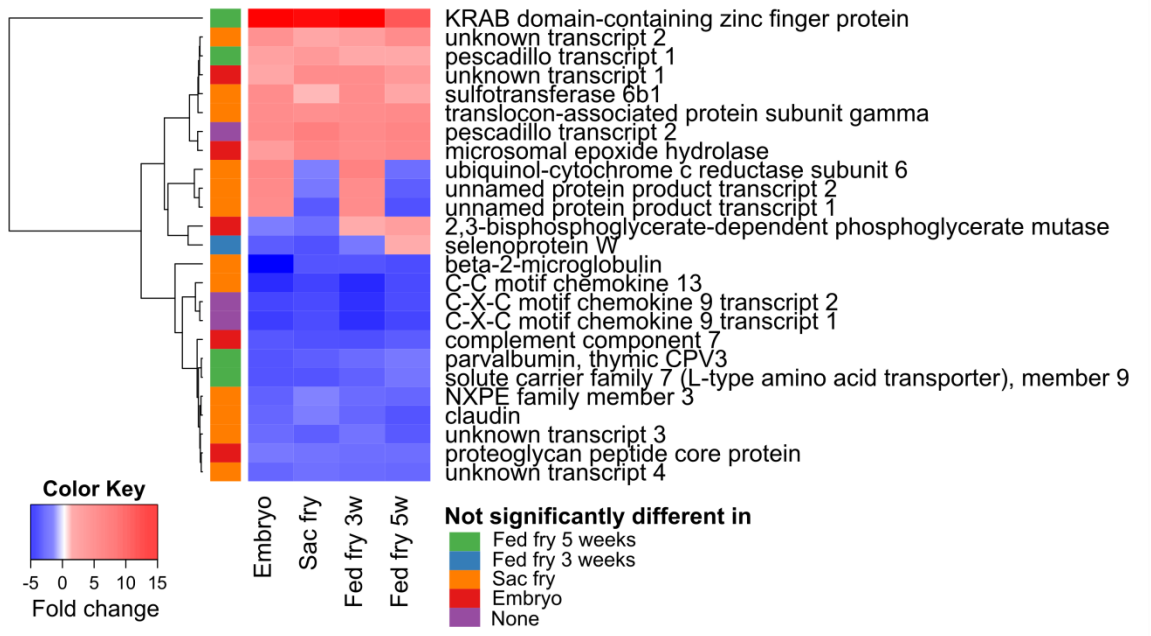
The highest numbers of differentially expressed transcripts were observed in the feeding fry studies; 1015 and 934 transcripts three and five weeks into feeding respectively (Figure 6.1). In comparison, relatively low numbers of transcriptional differences were detected in the embryo (299 transcripts) and sac fry (168 transcripts) studies. Most shared differences (132 transcripts) occurred between the embryo and 3 week fed fry, followed by 98 common transcripts identified between the two feeding fry experimental groups.

Figure 6.1 A comparison of the differentially expressed transcripts between wild and domesticated pure crosses identified by T-tests (corrected *p*<0.1) in the various life stages.



There were 26 transcripts that were shared amongst a minimum of three life stages. A single transcript annotated *C-C motif chemokine, other* and consistently down regulated (>2 fold) in the domesticated fish in 3 life stages has failed quality filtering in the fed fry 3 weeks experimental groups. Hence, the remaining 25 transcripts that have passed quality filtering in all the experiments are presented with their annotation (Figure 6.2). Hierarchical clustering revealed that the majority of these differentially expressed transcripts had consistent direction of expression across life stages when domesticated and wild stocks were contrasted. Hence, two major clusters were formed; one consisting of genes that are up-regulated and another comprising of genes that were down- regulated in the domesticated origin fish compared to wild origin fish. Two smaller groupings were also apparent. One cluster containing *2,3-bisphosphoglycerate-dependent phosphoglycerate mutase* and *selenoprotein W*, whose profile indicated enhanced expression in domesticated fish compared to wild fish transitioning at external feeding stage. The other cluster contained two genes with no annotation available and the *ubiquinol-cytochrome c reductase subunit 6*. These genes were up-regulated in domesticated embryo and fed fry (3 weeks), but down regulated in sac and 5 week fed fry.

Figure 6.2 Hierarchical clustering of the fold changes of the transcripts that were differentially expressed between wild and domesticated pure crosses in at least three life stages.



6.3.2 Functional analysis

Approximately 62% of the sequences associated with probes that have passed quality filtering had KO numbers assigned to them, out of which about 26% were unique genes. The functional analysis packages used, returned a smaller number of pathways whose members were either up or down regulated (Figure 6.3), than bi-directionally perturbed pathways (Figure 6.4). Yet, the number of genes identified by all three packages was larger in the former. Biological pathways that were supported by all three functions are presented in Table 6.2 (up/down regulated) and Table 6.3 (bi-directional). The directions of the pathways were largely consistent across life stages. Where disagreement occurred, it concerned the *5 weeks fed fry* experimental group (Table 6.2). A very small number of uni-directionally perturbed pathways were detected in the embryos compared to the other life stages (Figure 6.3) reducing the chance of finding one directionally perturbed pathways that are present in all four life stages. Major biological functions consistently affected in the other three life stages involved the *immune* and *nervous systems* (Table 6.2). Bi-directionally perturbed pathways were mainly involved in *signaling* and the *chemokine signaling pathway* was commonly perturbed in all four life stages (Table 6.3).

Figure 6.3 A comparison of up and down regulated pathways (1d) between wild and domesticated pure crosses identified by *gage*, *roast* and *romer*.

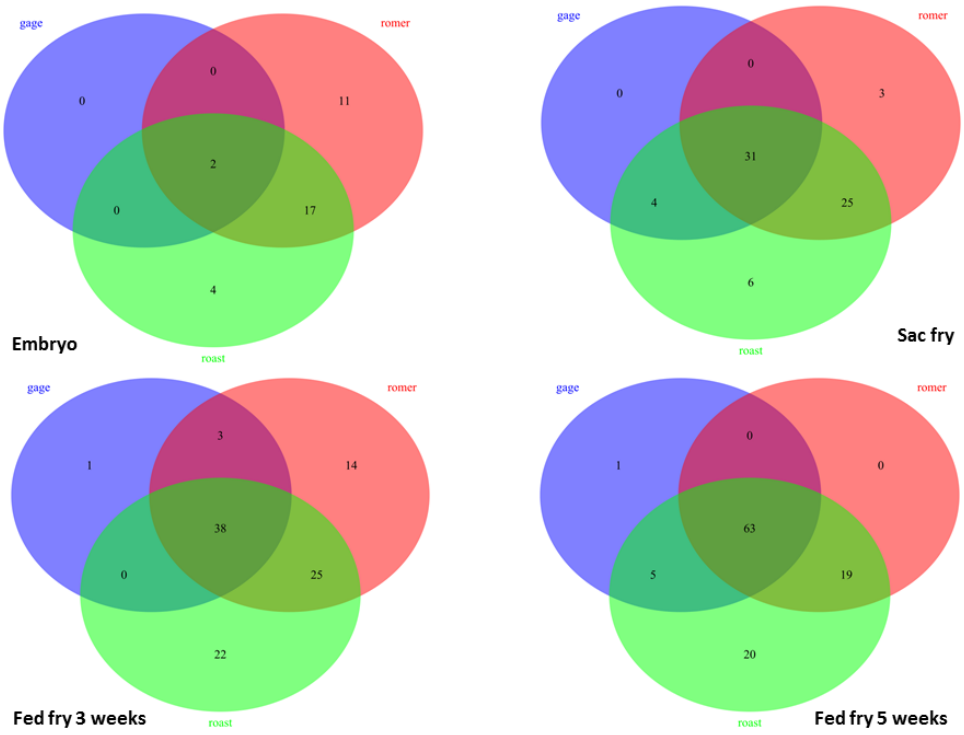


Figure 6.4 A comparison of differentially expressed pathways whose genes showed bidirectional change (2d/Mixed) between wild and domesticated pure crosses according to *gage*, *romer* and *roast* functions.

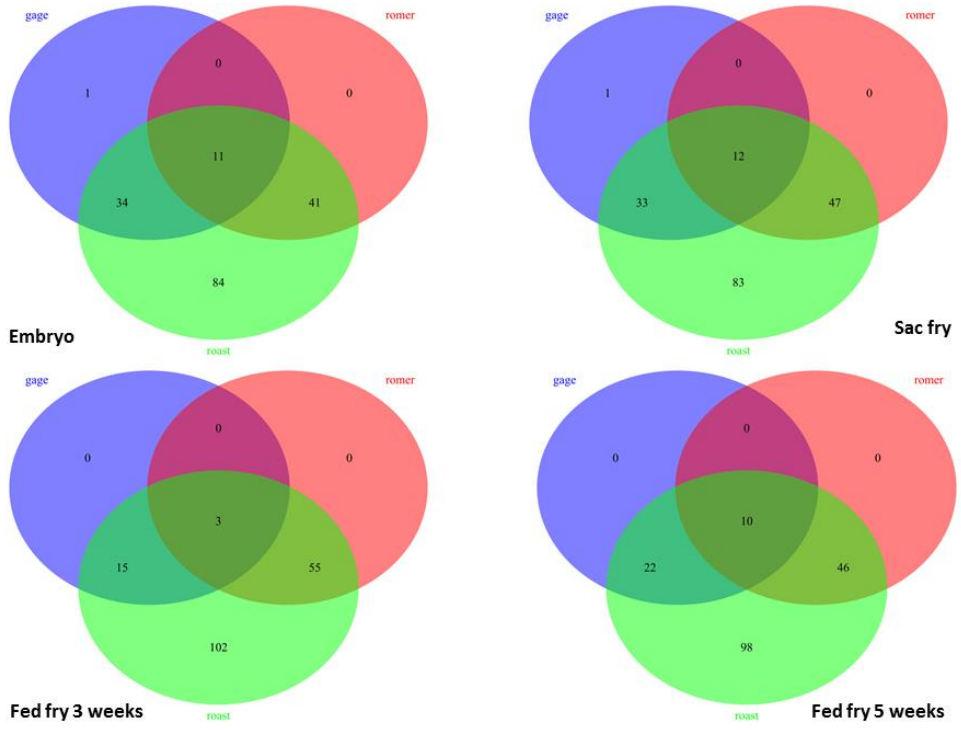


Table 6.2 Up and down regulated pathways (1d) that were significantly differentially expressed according to all three functional analysis functions. Direction of change is shown as domesticated compared to wild and directions that are inconsistent between life stages are presented in bold.

Kegg group	Sub-group	Pathway	Embryo	Sac fry	Fed fry 3w	Fed fry 5w
Cellular Processes	Cell motility	Regulation of actin cytoskeleton			Down	
	Cellular community	Focal adhesion				Up
	Transport and catabolism	Phagosome		Down	Down	
		Lysosome			Up	
		Endocytosis			Down	
		Peroxisome				Up
Environmental Information Processing	Signal transduction	Phosphatidylinositol signaling system	Down	Down		
		NF-kappa B signaling pathway		Down	Down	Down
		Jak-STAT signaling pathway		Down	Down	Down
		Hippo signaling pathway - fly		Up		
		VEGF signaling pathway			**Down**	**Up**
		TNF signaling pathway				Down
	Signaling molecules and interaction	Cytokine-cytokine receptor interaction		Down	Down	Down
		Cell adhesion molecules (CAMs)			Down	
		Neuroactive ligand-receptor interaction				Down
		ECM-receptor interaction				Up
Genetic Information Processing	Folding, sorting and degradation	Protein export		Up		
		Protein processing in endoplasmic reticulum		Up		
		Proteasome				Down
	Replication and repair	DNA replication		Up		
		Base excision repair		Up		
		Nucleotide excision repair		Up		
		Mismatch repair		Up		
	Transcription	Spliceosome		**Up**		**Down**
		RNA polymerase			Up	
	Translation	Ribosome		**Up**	**Up**	**Down**
		Ribosome biogenesis in eukaryotes		Up	Up	
		RNA transport		Up	Up	
		Aminoacyl-tRNA biosynthesis		**Up**		**Down**

Metabolism	Amino acid metabolism	Lysine degradation		Up		
		Glycine, serine and threonine metabolism				Up
		Valine, leucine and isoleucine degradation				Up
		Arginine and proline metabolism				Up
		Histidine metabolism				Up
		Tryptophan metabolism				Up
	Carbohydrate metabolism	Galactose metabolism			Up	Up
		Amino sugar and nucleotide sugar metabolism			Up	Up
		Glycolysis / Gluconeogenesis				Up
		Citrate cycle (TCA cycle)				Up
		Pentose phosphate pathway				Up
		Pentose and glucuronate interconversions				Up
		Fructose and mannose metabolism				Up
		Starch and sucrose metabolism				Up
		Pyruvate metabolism				Up
		Glyoxylate and dicarboxylate metabolism				Up
		Propanoate metabolism				Up
	Energy metabolism	Oxidative phosphorylation	**Down**	**Down**		**Up**
		Methane metabolism				Up
		Carbon fixation in photosynthetic organisms				Up
		Carbon fixation pathways in prokaryotes				Up
	Lipid metabolism	Biosynthesis of unsaturated fatty acids			Up	Up
		Sphingolipid metabolism			Up	
		Fatty acid elongation				Up
		Fatty acid degradation				Up
		Steroid biosynthesis				Up
		Primary bile acid biosynthesis				Up
		Steroid hormone biosynthesis				Up
		Glycerolipid metabolism				Up
		Glycerophospholipid metabolism				Up
	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism				Up
	Metabolism of other amino acids	Glutathione metabolism				Up
	Nucleotide metabolism	Pyrimidine metabolism			Up	

Table 6.3

	Xenobiotics biodegradation and metabolism	Drug metabolism - cytochrome P45			**Down**	**Up**
		Metabolism of xenobiotics by cytochrome P45				Up
Organismal Systems	Development	Osteoclast differentiation			Down	Down
	Digestive system	Fat digestion and absorption			Up	Up
		Bile secretion			Up	
		Mineral absorption			Up	
		Carbohydrate digestion and absorption				Up
		Protein digestion and absorption				Up
		Vitamin digestion and absorption				Up
	Endocrine system	PPAR signaling pathway				Up
		Insulin signaling pathway				Up
		Adipocytokine signaling pathway				Up
	Environmental adaptation	Circadian entrainment		Down		Down
	Immune system	Chemokine signaling pathway		Down	Down	Down
		Complement and coagulation cascades		Down	Down	
		B cell receptor signaling pathway		Down	Down	
		Hematopoietic cell lineage		Down		
		Toll-like receptor signaling pathway			Down	Down
		Natural killer cell mediated cytotoxicity			Down	Down
		Fc gamma R-mediated phagocytosis			Down	Down
		T cell receptor signaling pathway			Down	
		Fc epsilon RI signaling pathway			Down	
		Antigen processing and presentation				Down
		NOD-like receptor signaling pathway				Down
	Nervous system	Retrograde endocannabinoid signaling		Down	Down	Down
		Glutamatergic synapse		Down	Down	Down
		GABAergic synapse		Down	Down	Down
		Synaptic vesicle cycle		Down	Down	
		Cholinergic synapse		Down		
		Long-term potentiation			Down	
		Serotonergic synapse			Down	
		Phototransduction		Down	Down	
		Olfactory transduction		Down		

Kegg group	Sub-group	Pathway	Embryo	Sac fry	Fed fry 3 w	Fed fry 5w
Cellular Processes	Cellular community	Focal adhesion	2D			
Environmental Information Processing	Signal transduction	Hippo signaling pathway - fly	2D			
		NF-kappa B signaling pathway		2D		2D
		PI3K-Akt signaling pathway		2D		
	Signaling molecules and interaction	Cell adhesion molecules (CAMs)			2D	2D
Metabolism	Carbohydrate metabolism	Starch and sucrose metabolism	2D			2D
		Galactose metabolism	2D			
		Glycolysis / Gluconeogenesis				2D
Organismal Systems	Development	Osteoclast differentiation		2D		2D
	Digestive system	Fat digestion and absorption		2D		
	Endocrine system	GnRH signaling pathway	2D			
		Estrogen signaling pathway	2D			
		Prolactin signaling pathway	2D			
		Thyroid hormone synthesis			2D	
		Adipocytokine signaling pathway				2D
	Immune system	Chemokine signaling pathway	2D	2D	2D	2D
		Toll-like receptor signaling pathway	2D	2D		
		Fc epsilon RI signaling pathway	2D	2D		
		Natural killer cell mediated cytotoxicity		2D		2D
		NOD-like receptor signaling pathway		2D		
		Antigen processing and presentation				2D
		Hematopoietic cell lineage				2D
	Nervous system	Long-term potentiation	2D			
		Retrograde endocannabinoid signaling		2D		
		Cholinergic synapse		2D		
		Dopaminergic synapse		2D		

Table 6.3 Differentially perturbed pathways consisting of genes that are up and down regulated within the same pathway (2d/Mixed) and that were identified by all three functional analysis methods.

6.3.3 Heritability

In line with the statistical analysis that excluded hybrid data, inclusion of data from hybrids also showed that most shared differences occurred when either the families (embryo and fed fry 3 weeks) or the life stages (fed fry 3 and 5 weeks) were shared between experimental groups (Figure 6.5A). This was also reflected in the overlap of the heritability categories between life stages (Figure 6.5 B-D).

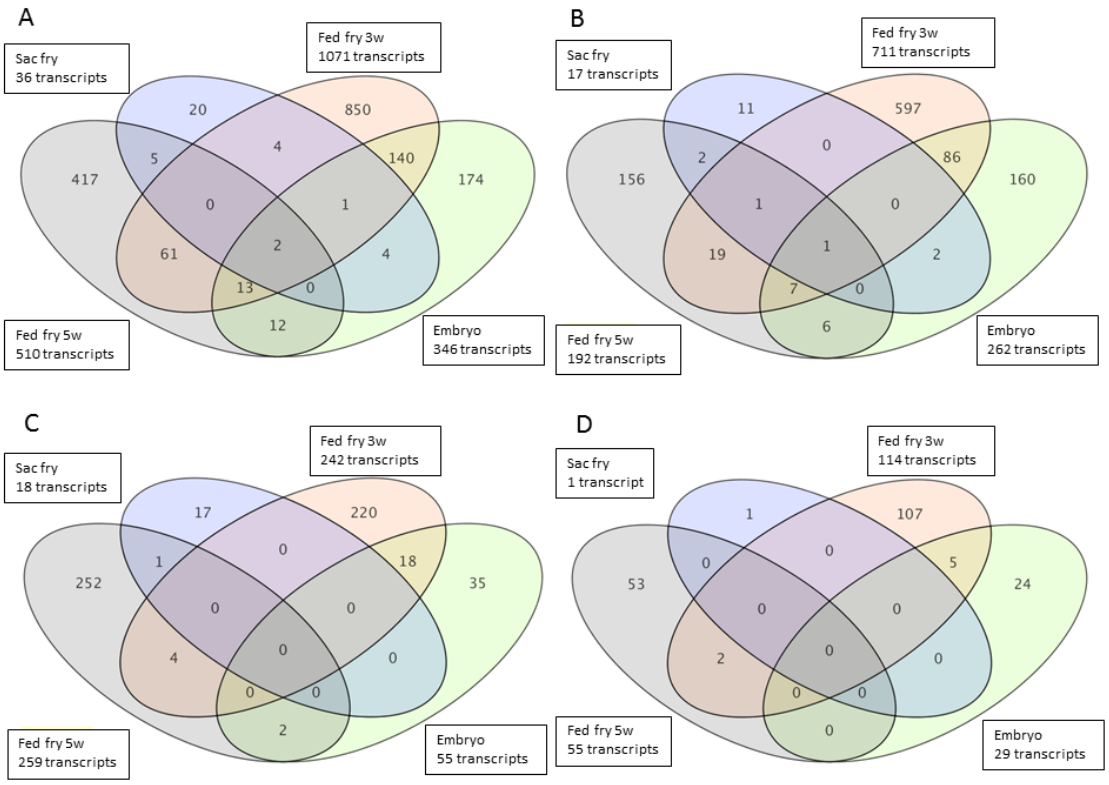


Figure 6.5 A. A comparison of the differentially expressed transcripts identified between the crosses in the various life stages by 1-way ANOVA (corrected p<0.1). B. Differentially expressed transcripts under parental influence. C. Differentially expressed transcripts exhibiting maternal effects. D. Differentially expressed transcripts exhibiting paternal effects

A total of 243 transcripts were detected as differentially expressed across the crosses in at least two life stages. Out of these, 139 had KEGG annotations assigned to them, of which 121 were unique KOs. A number of genes (43) were excluded as they were not associated with a pathway from the five main functional groups considered leaving 78 unique genes that were plotted for heritability (δ/α) (Figures 6.6-6.10) and additive parameters (α) (Figures 6.11-6.15). The additive parameter, indicating the direction of

the expression change between wild and domesticated pure crosses was very consistent across life stages, to an extent where scatter points frequently overlapped between the embryo and 3 week fed fry life stages (Figures 6.11-6.15). Similarly, the heritability of gene expression was found to be consistent across life stages, especially when the same families were used (Figures 6.6-6.10). There were less detectable transcriptomic differences in the *sac fry* and *5 weeks fed fry* comparison. Among the genes considered, additivity was the most pronounced mode of inheritance, ranging from 31% to 59%, exhibited by embryos and fed fry 5 weeks respectively (Table 6.4). A large proportion of genes belonging to most functional groups exhibited intermediate hybrid expression. Additivity described the vast majority of genes belonging to the *immune system*, *signaling molecules and interactions* and *folding, sorting and degradation* pathways, whereas it was least characteristic to *cell growth and death* and *cell motility* functions. Maternal dominance was also prominent affecting 19-45% of the genes. Although in the majority of the life stages it ranged between approximately 19-33%, only increasing to 45% in the sac fry. Maternal dominance was obvious from the heritability of genes associated with the *environmental information processing* functional group and the *endocrine system*, whereas genes with *immune* functions only appeared to be maternal dominant, when the eggs originated from domesticated dams (Figure 6.6).

Figure 6.6-6.10 A comparison of the heritability of the differentially expressed genes identified in at least two life stages. It is calculated for both reciprocal hybrids and grouped according to functions.

Figure 6.11-6.15 A comparison of the additive parameters of the differentially expressed genes identified in at least two life stages and their representation by functional groups.

Figure 6.6

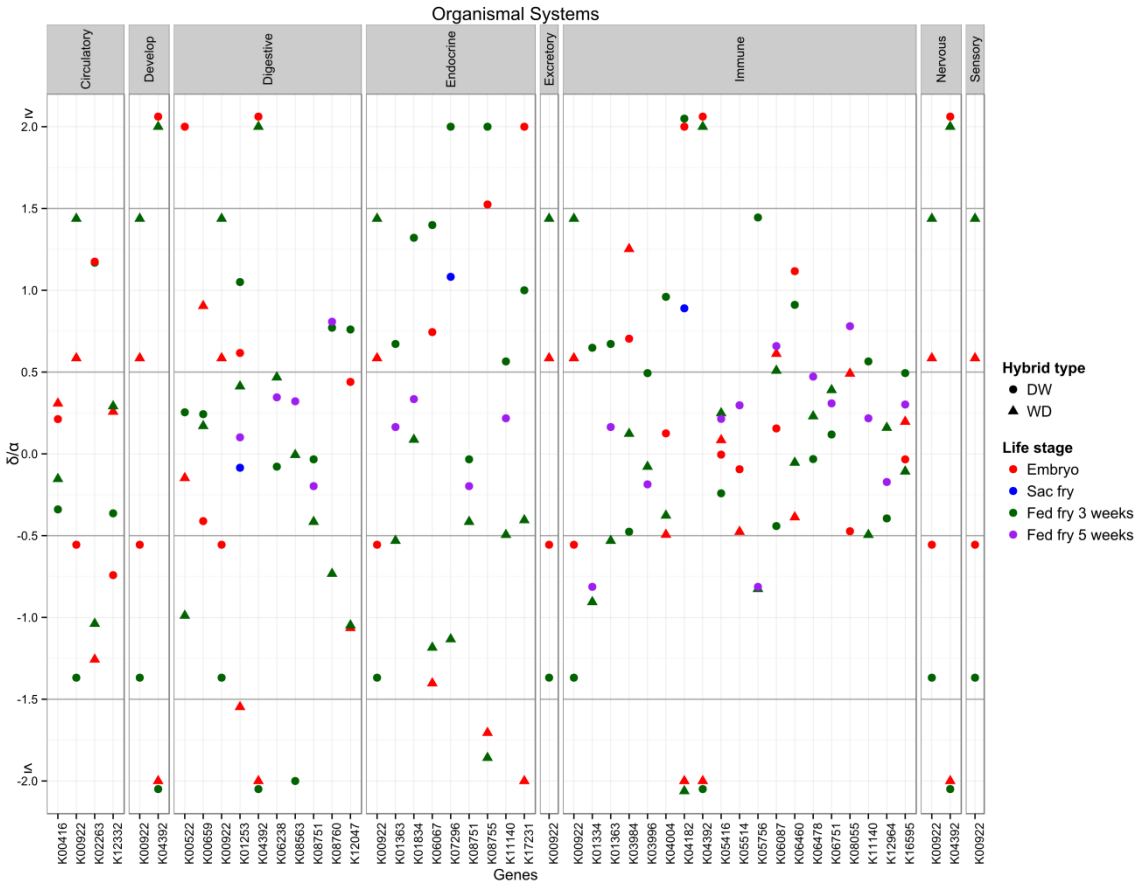


Figure 6.7

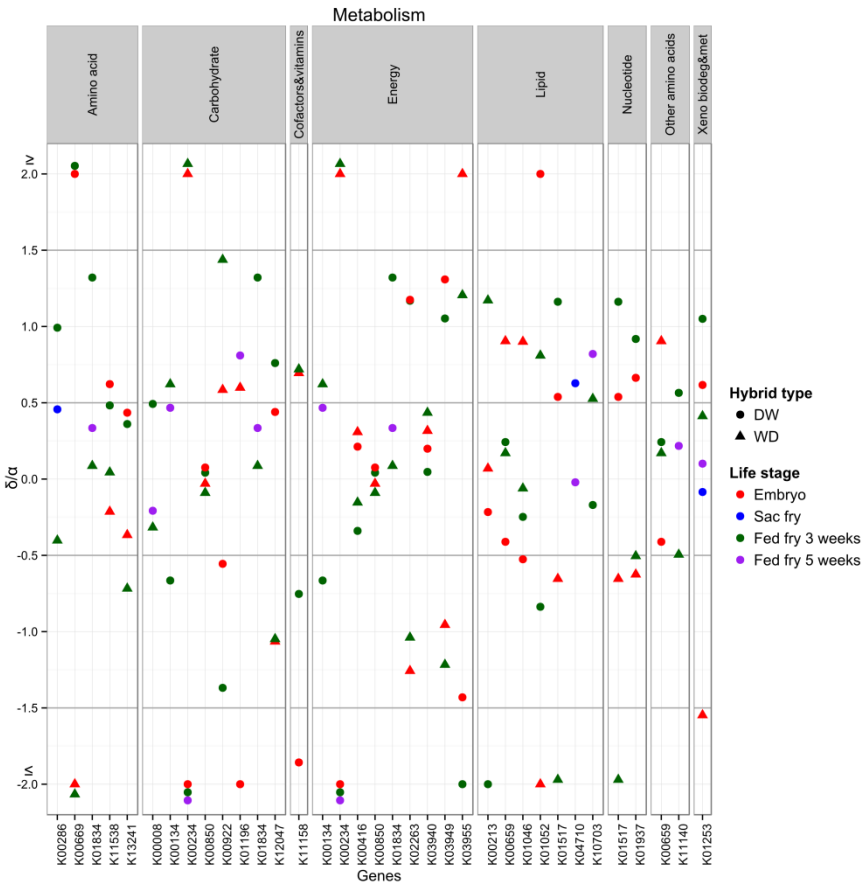


Figure 6.8

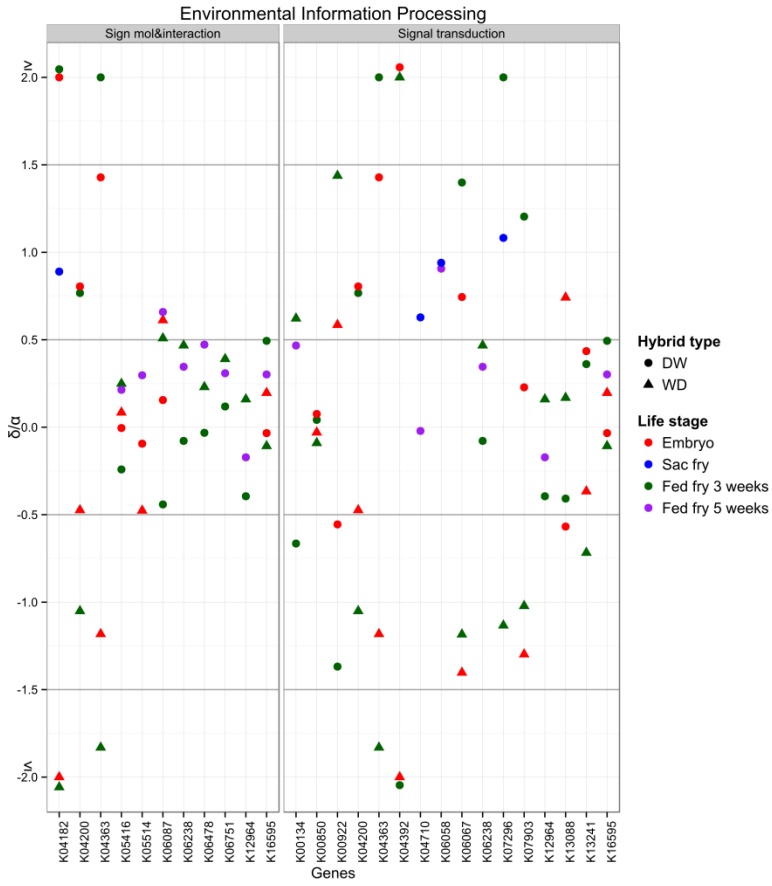


Figure 6.10

Figure 6.9

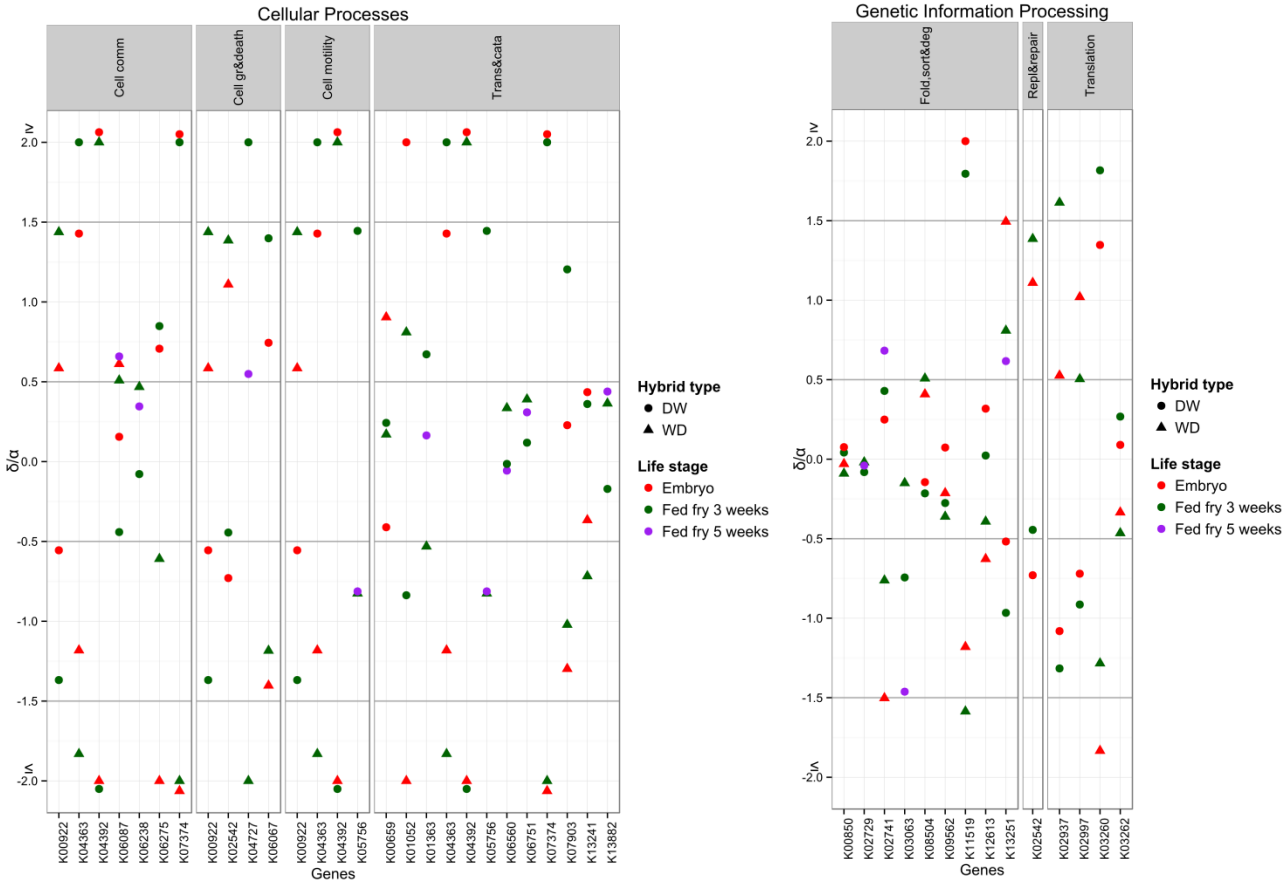


Figure 6.11

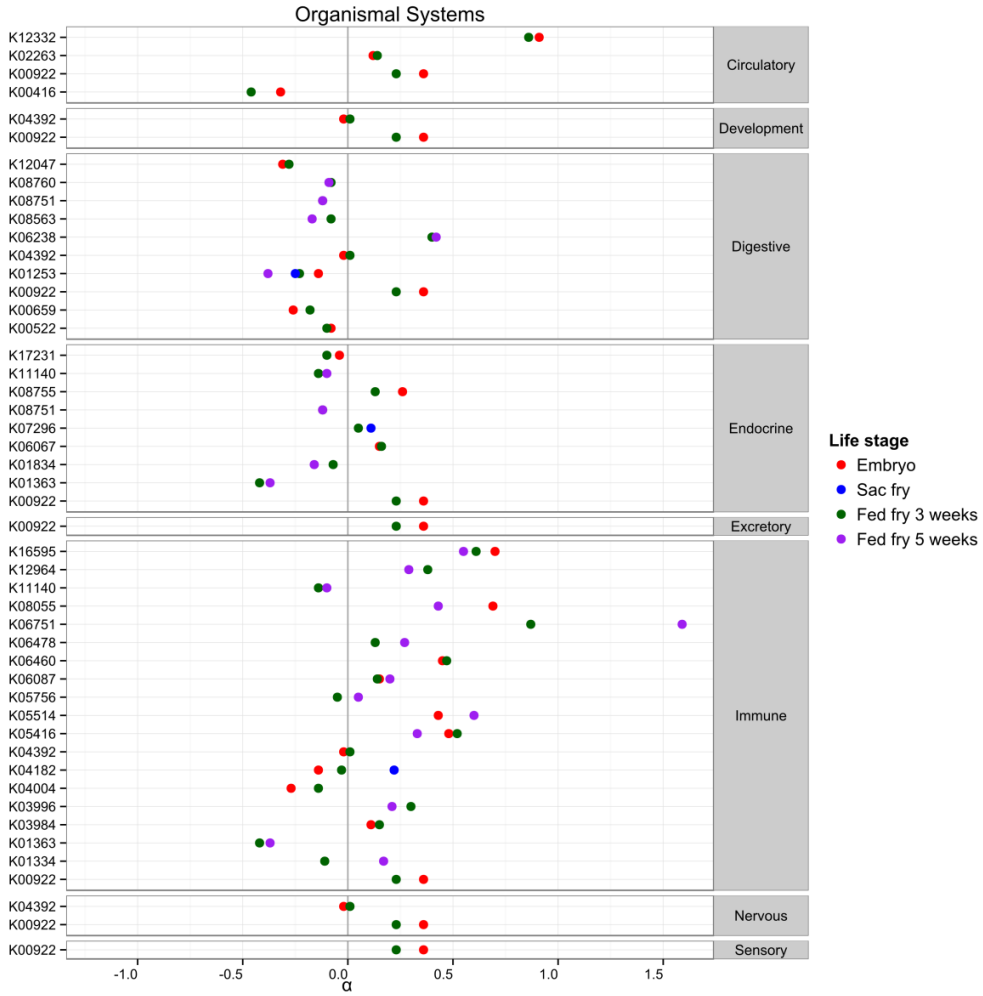


Figure 6.12

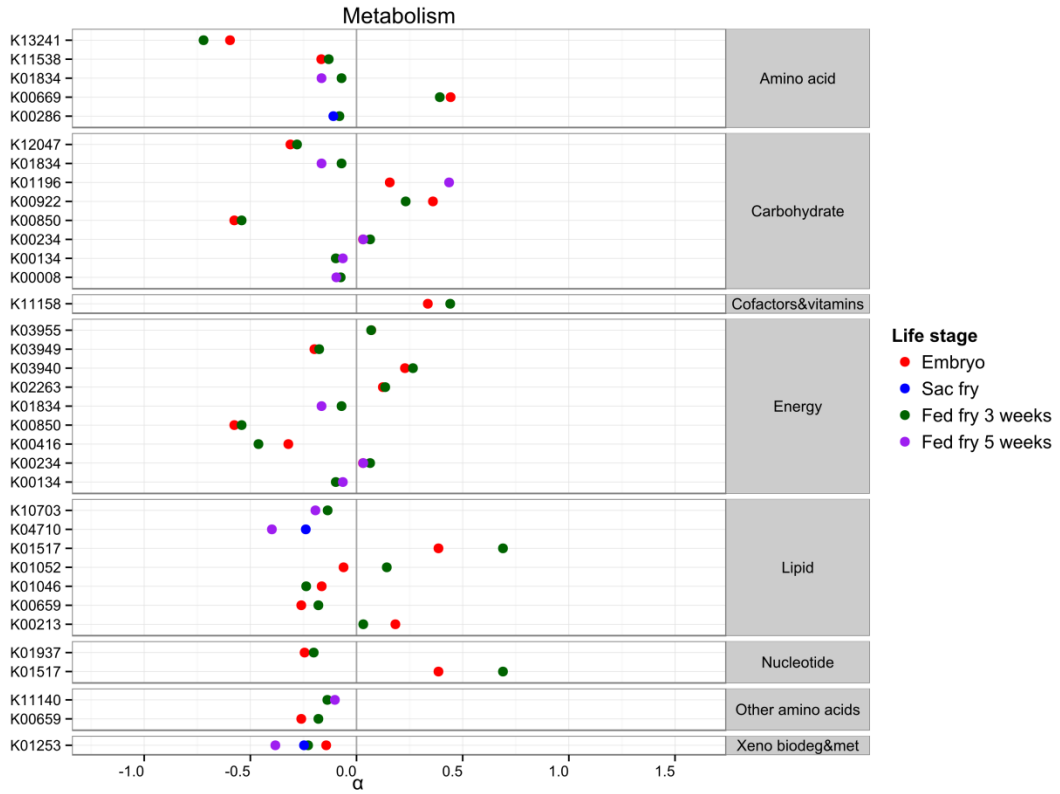


Figure 6.13

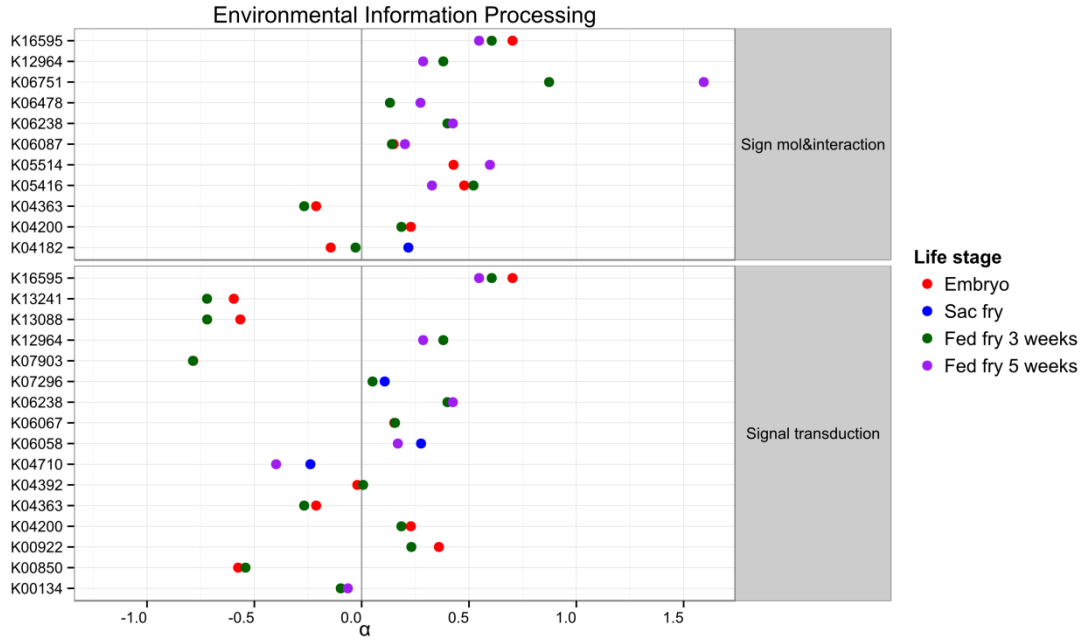


Figure 6.14

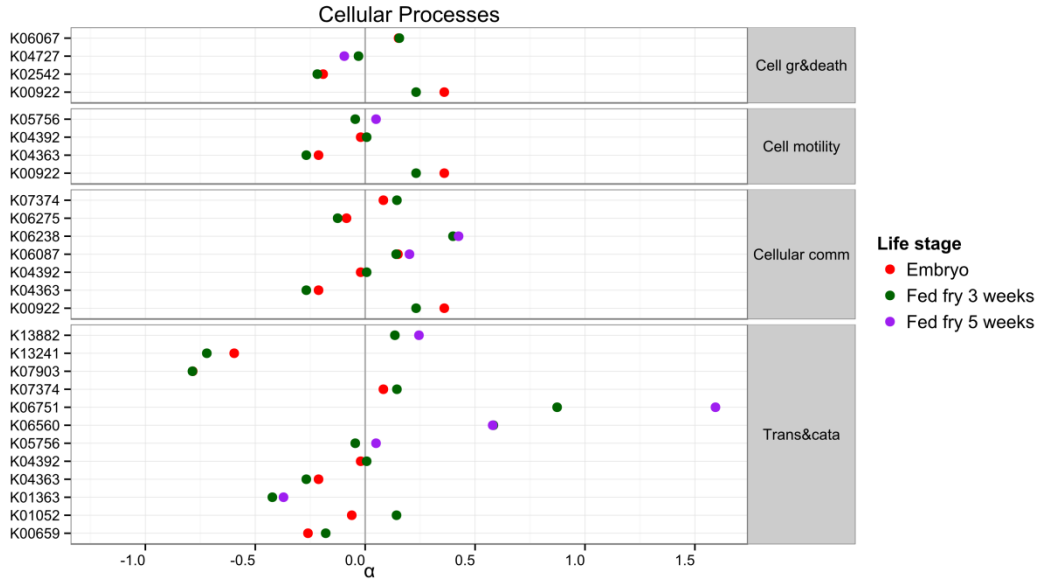


Figure 6.15

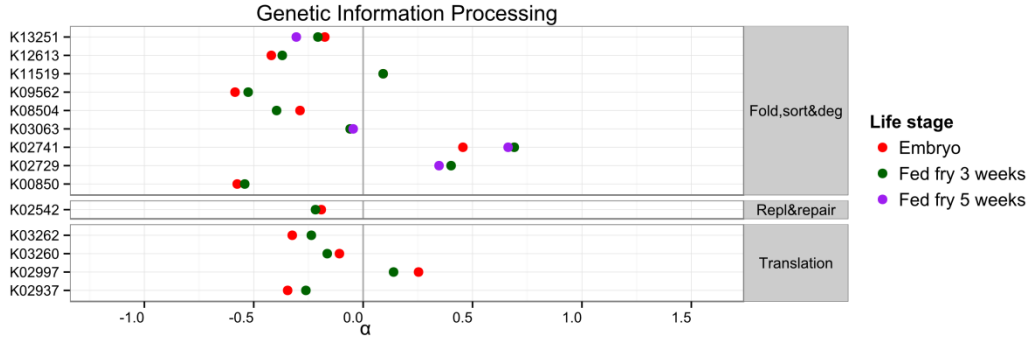


Table 6.4 A heritability comparison based on the δ/α of the genes that were differentially expressed in more than one life stage.

	Wild over-dominant		Wild dominant		Additive		Domesticated dominant		Domesticated over-dominant		Total number of genes
	WD	DW	WD	DW	WD	DW	WD	DW	WD	DW	
Embryo	26.19%	7.14%	19.05%	13.10%	30.95%	36.90%	17.86%	20.24%	5.95%	22.62%	84
Sac-fry		0.00%		0.00%		54.55%		45.45%		0.00%	11
Fed fry 3 weeks	14.68%	7.34%	22.02%	10.09%	42.20%	40.37%	15.60%	25.69%	5.50%	16.51%	109
Fed fry 5 weeks		2.04%		6.12%		59.18%		32.65%		0.00%	49

6.4 Discussion

6.4.1 Overview

Feeding fry life stages revealed more transcriptomic differences between wild and domesticated stocks than the embryo or yolk sac stages. There are several possible explanations for this, and the reduced number of detected genes in these earliest life stages is most likely the result of the combination of them. First, considering that the microarray was designed largely from published EST sequences from later life stages, i.e. feeding fry onwards, the probe set may lack some power for detecting transcriptomic differences characteristic to early life stages. Second, the organ to body/egg size ratios may have masked some of the differences present between wild and domesticated fish. Third, with the initiation of exogenous feeding a number of metabolic pathways are activated (Mennigen et al., 2013) possibly revealing additional differences between the stocks. Indeed all of the functional differences unique to the feeding stage are involved in metabolism; mainly lipid and carbohydrate, or have digestive or endocrine functions. Interestingly, only approximately 10% of the statistically significant transcriptomic differences overlapped between the two feeding stages (3 week and 5 week feeding) and in addition, considerably more metabolic differences were apparent in the 5 weeks fed fry cf. 3 weeks fed fry. This suggests that the additional transcriptomic differences are either less robust or are not yet present at 3 weeks post feeding. Considering that whole individuals were used, it is plausible that the size of the organs responsible for these metabolic differences may be below statistically significant detection level at 3 weeks but not at 5 week post start feeding. It is also possible that a longer exposure to the commercial diet was necessary to trigger a wider range of metabolic differences between wild and domesticated stocks, thus only revealing them at 5 week post feeding. Regardless of the reason, all of the metabolic pathways identified were up regulated in domesticated fish cf. wild fish in both life stages. Since farmed salmon stocks are selected for growth under commercial conditions, which includes the consumption of artificial diets, altered feeding behaviour and/or feed utilization could induce metabolic differences between

wild and domesticated salmon. Indeed, greater feed consumption and more efficient feed utilization have been reported for Atlantic salmon that had been selected for growth for 5 generations, when compared with its wild counterparts (Thodesen et al., 1999).

In contrast to the limited overlap within the two exogenous feeding stages, a third of the differences that were detected in embryos were also found in the fry fed (3 weeks) experimental group. In these experiments the embryos and feeding fry (3 weeks) consisted of pools of the same families; i.e.: originating from the same parents. This suggests that the genetic background of the parents may have considerable impact on the differences commonly detected. It has been suggested that due to the genetic architecture of the different salmon stocks, parallel transcriptomic changes in response to domestication are scarce at gene and even at pathway level, but rather manifest in shared functional groups (Roberge et al., 2006). Based on the scale of consistencies between the vastly different life stages (embryo vs 3 weeks fed fry) where the same families were studied, but overall limited similarities between experiments of the same strains, it is possible that it is not only the selection regime acting on different strains, but also includes individual genetic differences that effect the genes identified as differentially expressed in response to domestication.

6.4.2 Universal gene specific differences

Differentially expressed transcripts that are shared between multiple life stages are the most likely to be robust and potentially diagnostic between the wild and domesticated stocks studied. The analysis focused on the differences that were common in at least three life stages, as due to the experimental design, limiting the search to two of them would not have fully eliminated life stage specific and family background biases. For example, the exogenous feeding stage is represented twice in the comparison (3 weeks & 5 weeks since start feeding) and thus the shared differences could have been purely environmental, i.e.: differences between wild and domesticated fish in commercial salmon feed utilization. In addition the embryo and 3 week fed fry

experimental groups have utilized the same families and thus the detected similarities may have been due to the genetic architecture of the families used. Sac fry and 5 week fed fry have also shared cohorts. In this case, similarities are less pronounced, since although the same parents were used a larger number of families (10) were produced and randomly sampled. This has led to a less balanced family design than in case of the embryo and 3 week fed fry experimental groups, where equal numbers of individuals from exactly the same two families were pooled. Although using a larger number of families and a less balanced design introduced variation and has reduced the number of detectable differences, it has also diluted family effects.

Overall, the directions of change of the transcriptomic differences detected between domesticated and wild fish were consistent across life stages. Genes that were up regulated in at least three life stages of domesticated fish compared to wild fish were largely involved in growth and/or development (*KRAB domain-containing zinc finger protein*, *pescadillo*, *sulfotransferase 6b1*, *translocon-associated protein subunit gamma*), whereas most down regulated genes had immune (*beta-2-microglobulin*, *chemokines, complement component 7*, *parvalbumin*, *thymic CPV3*, *claudin*) and/or cell adhesion functions (*proteoglycan peptide core protein*, *claudin*). The clustering of the shared differentially expressed genes was also indicative of life stage and family specific differences. Based on the expression patterns of the genes transcribing for *2,3-bisphosphoglycerate-dependent phosphoglycerate mutase* and *selenoprotein W* and the metabolic functions of these proteins, it is likely that the increase in expression of these genes (domesticated vs wild) is associated with the initiation of exogenous feeding of the fish. According to its KEGG classification *2,3-bisphosphoglycerate-dependent phosphoglycerate mutase* is involved in *glycolysis / gluconeogenesis* by catalysing the interconversion of *2-phosphoglycerate* and *3-phosphoglycerate*, while *selenoprotein W* synthesis requires selenium; an essential nutritional trace element (Zhang et al. 2012) that is supplemented in fish feed (Lall 2008). *Ubiquinol-cytochrome c reductase subunit 6* and two other proteins with unknown function showed

expression indicative of the genetic background of the families. This protein is involved in oxidative phosphorylation taking place in the primarily maternally inherited mitochondria (Suzuki et al. 1990), which may explain the differences detected between different cohorts.

6.4.3 Functional differences

Overall, a smaller number of uni-directionally perturbed pathways than bi-directionally perturbed pathways were detected between pure domesticated and wild fish. However, there was a greater overlap between the results of the different packages for this group, compared to the bi-directional analysis. There was no contradiction between the different methods in identifying the direction of the perturbation of the differentially expressed pathways. Moreover, the direction of change was also mainly consistent across life stages. Where discrepancies occurred, it involved the 5 weeks fed fry experimental group; namely the up regulation of *VEGF signaling*, *oxidative phosphorylation*, *drug metabolism – cytochrome P45* pathways, and the down regulation of *RNA transcription* and *translation* pathways in the pure domesticated fish compared to pure wild fish. *VEGF* plays a role in angiogenesis, bone formation, hematopoiesis, wound healing, and development (Duffy et al., 2000), whereas *oxidative phosphorylation* is involved in *energy metabolism*, both of which may be associated with increased oxygen demand and increased overall metabolism of the domesticated feeding fry, compared to non-feeding life stages. Moreover, down regulated mRNA transcription and translation pathways detected in the 5 weeks fed fry contradict a large number of *genetic information processing* pathways that were found to be up regulated in the sac fry. Although supported by a smaller number of pathways, the same trend was observed in the 3 weeks fed fry group too. A literature search did not uncover any plausible explanation for the down regulation of the *genetic information processing* pathways in the 5 week fed domesticated fish compared to the wild stock. The observation appears somewhat counterintuitive as these functions were up regulated in earlier life stages, consistent with hypothesised increased protein synthesis and growth in domesticated fish. Indeed, individual genes of the *genetic*

information processing that were statistically significantly different between domesticated and wild fish showed consistent expression across all life stages and were mainly up regulated in the domesticated cross. This discrepancy between pathway and gene analyses is possible, since gene set tests in general, establish correlation between functional groups and phenotype by detecting small but coordinated changes in gene expression, regardless of statistical status of individual genes (Luo et al., 2009; Tarca et al., 2013; Wu et al., 2010). Thus, it seems that there were a small number of statistically significant up regulated *genetic information processing* genes in the 5 week fed domesticated fish, while an individually modest, but overall detectable reduction of the expression of the genes belonging to this functional group has also been detected. Combined with the large number of up-regulated metabolic pathways, characteristic to the domesticated 5 week fed fry, a possible and unsettling explanation is that the increased amount of metabolic transcripts in these samples reduced the proportion of other transcripts and this subtle change was reflected in the gene set analyses methods. *Genetic information processing* occurs in all cells and mediated by mRNA that is being compared during microarray analysis, and hence may serve as a possible explanation, if this function really was profoundly affected by the overall composition of the RNA sample.

Apart from the *genetic information processing* group, all other functions were consistently regulated across life stages and included down regulated *immune* and *nervous system* and *environmental information processing* pathways in the domesticated fish. Selection pressures operating on wild populations and those acting during domestication greatly vary (Price, 2002).Thus, genetic variants favoured under natural and culture niches will also differ (Werf et al., 2009). Due to traits having different significance under different environmental conditions, the optimal allocation of resources is likely to vary between wild and domesticated animals. Since growth is often heavily selected for in domesticated populations, a trade-off with another high energy demanding trait, such as the immune function, has been proposed to occur in

some domesticated animals (Rauw, 2012). Indeed, parallel to down-regulated immune function of domesticated, compared to wild fish, up regulated carbohydrate and lipid metabolism, as well as digestive and endocrine system were observed in domesticated feeding fry, and a number of growth/development related genes were also identified in feeding and pre-feeding life stages. As part of domestication selection, the reduction of information acquisition and processing systems, including those involving sensory organs and synapses with transmitter substances for information processing, has also been proposed (Hemmer, 1990). In support of this theory, most nervous system pathways that were found to be down regulated in domesticated fish in this study are related to sensory or synaptic functions. Moreover, increased aggression and reduced predator avoidance of domesticated Atlantic salmon have also been reported (Einum and Fleming, 1997).

A comparable study investigating parallel changes of Atlantic salmon populations in response to domestication conducted on whole sac fry reported similar functional classes, as reported here. These included differentially expressed transcription regulation, protein synthesis, immunity and digestion between domesticated and wild crosses (Roberge et al., 2006). Despite the different microarray designs it would have been interesting to include the Roberge experiment in our comparison; however the microarray data this work is based on have not been made publicly available.

6.4.4 Heritability

Differentially expressed transcripts common in at least three life stages were rarely observed, when hybrids were included in the statistical analysis. This reflects the overall low number of detectable differences recorded for the sac fry and 5 weeks fed fry experimental groups. While the number of differentially expressed transcripts increased with reciprocal hybrids present (analysed for embryo and 3 weeks fed fry groups), the number decreased when only one hybrid group was added (sac and 5 weeks fed fry analyses), compared to the numbers when only pure crosses were considered. Since hybrids expression levels more closely resembled those of their

maternal pure cross, it is possible that by adding only one hybrid group, the mean per transcript expression, against which variance is being measured, shifted thereby reducing the variance to a non-significant level. Including the second reciprocal hybrid is likely to have counter balanced this shift.

When individual genes were compared the additive parameter was vastly consistent, whereas the mode of heritability was fairly consistent across life stages. This was especially true for expression of the embryo and 3 weeks fed fry groups, further highlighting the importance of the genetic background of the families analysed. Among the genes considered, additivity was the most pronounced, affecting from 31% to 59% of the transcripts; exhibited by embryos and 5 weeks fed fry respectively. Intermediate hybrid gene expression described a large number of genes in most functional groups. This mode of inheritance was especially pronounced in genes belonging to the *immune system*, *signaling molecules and interactions* and *folding, sorting and degradation* pathways. The significance of additivity has been highlighted in other gene expression studies of divergent salmonid populations, including wild and domesticated Atlantic salmon (Debes et al., 2012), brook charr (Bougas et al., 2010) and dwarf and normal lake white fish (Renaut et al., 2009). Moreover, additive genetic variation has been emphasised in other important Atlantic salmon traits, such as fitness, survival (Ferguson et al., 2007; Dylan J Fraser et al., 2010), and growth and behaviour (Dylan J Fraser et al., 2010; Glover et al., 2009; Solberg et al., 2013a).

A maternal dominance effect was also prominent, affecting 19-45% of the transcripts studied, although in the majority of the life stages it ranged between approximately 19-33%, only increasing to 45% in the sac fry. The unusually high value for maternal dominance may reflect the presence of unabsorbed yolk-sac or be an overestimation due to the small number of differentially expressed transcripts detected in this life stage. Maternal dominance was the most pronounced characteristic of genes associated with the *environmental information processing* functional group and the *endocrine system*. In addition, some non-additive genes of the *immune* system

exhibited dominance for domesticated mothers only. Maternal effects of the transcriptome have been scarcely studied in salmonids. However, a study of wild and domesticated brook charr has reported that 40% of detected differentially expressed genes were governed by maternal dominance (Bougas et al., 2010). Maternal effects on other traits are known to be common in salmonids and are mainly associated with egg and nest quality (Green, 2008). Egg and alevin size and survival are also maternally influenced (Einum and Fleming, 2000, 1999; Houde et al., 2011; Skaala et al., 2012).

6.5 Conclusion

The major transcriptomic differences between the studied wild and domesticated stocks that were shared across life stages were related to *immune* and *nervous system* functions or belong to the *environmental information processing* biological function. The vast majority of these pathways were down regulated in the domesticated fish, compared to their wild counterparts. The data also revealed a set of genes that were up-regulated in the domesticated fish in multiple life stages. These were involved in growth and/or development. These findings are indicative of disruption to the natural allocation of resources, reduction of information acquisition and processing systems and possible loss of local adaptation to natural environments of the domesticated fish, especially concerning immune function and these could well have a negative impact on farmed escapees and their offspring.

More transcriptomic differences between wild and domesticated Atlantic salmon were identified in the exogenous feeding fry life stages. Studying this life stage may be desirable if it maximises the detectable differences between the stocks due to a more optimal organ to body ratio despite studying whole individuals. However, it can also be disadvantageous if the transcriptomic differences are triggered by the exposure of wild fish to aquaculture conditions and commercial diet. Resolution to the above may be provided by experiments involving natural settings and diet, and tissue specific gene expression analysis.

In order to draw general conclusions regarding the genetic differences induced by Atlantic salmon domestication, multiple wild and domesticated stocks must be studied. Considering that strong family effects are also apparent from this comparative study it would be advisable to use a large number of families in future studies. Utilizing a large number of families and multiple wild and domesticated stock minimise the false identification of differences that arose due to the genetic background of specific families/stocks, as of those of the true effects of domestication.

The analysis showed additivity and maternal dominance to be the main forms of inheritance manifesting the transcriptomic differences between wild and domesticated Atlantic salmon. Whether these differences persist over subsequent generations may be investigated by studying backcross hybrids.

Chapter 7 - General discussion and conclusions

There is a perception that the aquaculture industry constitutes a major threat to wild Atlantic salmon populations (WWF, 2001). One of the main concerns, the genetic interaction between farmed escapees, has been a long standing issue (Saunders, 1991; Taranger et al., 2015). Norway, where Atlantic salmon farming originated and which is now the World's largest producer of the species (FAO, 2015), has 62% of its surveyed rivers under moderate–to-high risk of experiencing genetic changes due to introgression of farmed salmon (Taranger et al., 2015). Considering the high cultural and economic value of the species, there are several projects, which either solely or as part of a wider study, address this issue from multiple aspects. For example, SALSEA (www.nasco.int/sas/salsea.htm) is concerned with monitoring of the migration and distribution of salmon at sea, whereas, Prevent Escape (/preventescape.eu/) seeks to reduce the number of escaped farmed fish from European aquaculture. Genimpact (www.imr.no/genimpact/en) was formed to review and discuss current knowledge available to assess the genetic impact of various aquaculture species and the objective of AquaTrace (https://aquatrace.eu/) is to develop tools for tracing and evaluating the genetic impact of farmed escapees. Norwegian initiatives, the project INTERACT and the QuantEscape knowledge platform, aim to elucidate the genetic differences between wild and domesticated Atlantic salmon and cod, and to quantify genetic effects of escaped farmed salmon on wild salmon respectively. This thesis is part of INTERACT and its contributions to understanding the genetic interaction between wild and domesticated Atlantic salmon are discussed below.

7.1 Transcriptomic differences between wild and domesticated Atlantic salmon

Microarray analysis revealed a number of biological functions that were consistently differentially perturbed across life stages, between the wild and domesticated stocks

studied. E*nvironmental information processing* and *immune* and *nervous system* functions were down regulated in the farmed compared to wild fish in multiple early life stages. The alteration of many of the affected pathways is likely to be a result of local adaptation to captivity (domestication selection), including reduced information acquisition and processing system, altered stress responsiveness and feeding behaviour. In agreement with the resource allocation theory proposed for domesticated livestock (Rauw, 2009), reduced immune function was coupled with increased expression of growth and development related pathways, such as increased expression of *genetic information processing, metabolism*, *digestive* and *endocrine systems,* characteristics of the domesticated fish. The indicators of the trade-off in favour of increased growth of the domesticated fish did not seem to be constant across life stages. A shift was observed from the increased expression of *genetic information processing* pathways in pre-first feeding progeny of domesticated fish to the increased expression of *metabolism*, *digestive* and *endocrine system* pathways following the initiation of exogenous feeding. The transition was such that the domesticated origin fish exhibited increased *genetic information processing* involving a large number of biological pathways in the sac fry stage, then in the feeding fry (3 weeks), this was reduced to a smaller number of pathways mainly affecting *transcription* and *translation*, whereas pathways indicating increased *metabolism*, *carbohydrates* and *lipids* in particular and the up-regulation of *digestive system* emerged in this life stage. By 5 weeks into first feeding a whole array of *metabolic*, *digestive* and *endocrine* pathways were found to be up-regulated in the domesticated fish and at the same time down-regulation of *transcription* and *translation*-related pathways was observed. During the transition from endogenous to exogenous feeding, salmonids initially exclusively rely on internal yolk-sac reserves. Following a first meal, a stage of mixed feeding occurs, during which exogenous food and the remaining yolk sac reserves are simultaneously utilised, before moving to completely exogenous feeding (Balon, 1986). Corresponding changes in the expression of metabolic genes occur, as the initiation of exogenous feeding alters gene expression by activating certain metabolic pathways, such the

glycolytic and fatty acid pathways (Mennigen et al., 2013). The increasing reliance on external feed may explain the gradual shift in the transcriptomic differences detected between wild and domesticated fish across life stages. The increased transcription of genes associated with the *digestive system* and/or involved in *metabolism* may be a result of increased feed intake and metabolism of hatchery diet that is characteristic of domesticated fish. In addition, selection for growth is likely to favour individuals with a more active *endocrine system*.

The most prominent *genetic information processing* pathways identified, *RNA transcription* and *translation,* are related to protein synthesis and growth, and, along with the other functions, have been indicated to be altered in response to domestication. Roberge *et al*. (2006) studied Canadian and Norwegian wild and domesticated Atlantic salmon strains and concluded that parallel changes occur at the level of biological functions, rather than genes or gene pathways. The functional classes Roberge and co-workers identified included *energy metabolism*, *transcription regulation*, *protein synthesis*, *immunity*, *muscle function* and *digestion*. Their study, conducted on fry at swim-up stage, found transcripts in *energy metabolism*, including *glycolysis* and *oxidative phosphorylation* to be expressed at lower levels in both farmed strains compared to wild counterparts. While *oxidative phosphorylation* was also found to be down-regulated in the domesticated embryo and sac fry in the current thesis, this function, along with *glycolysis/gluconeogenesis* and numerous additional *carbohydrate* and *lipid metabolism* pathways were up-regulated in domesticated feeding fry. A possible explanation for the change of direction in the regulation of pathways involved in, or associated with, *energy metabolism* is that wild and domesticated fish may process and/or allocate the various energy sources that are available in a given life stage differently. Endogenous feeding is an energetically closed system, since energy can only be sourced from the yolk, and it is partitioned primarily between growth and metabolism. During this period fish utilize free amino acids first, then mobilize yolk lipids and proteins. Following first feeding energy is

increasingly obtained from external sources, and is no longer largely partitioned only to growth and metabolism, but supports other energy demands and constraints, such as those associated with food foraging, egestion of faeces, locomotion, and social interactions (Kamler, 2007).

Another life-stage specific difference between wild and domesticated fish, highlighted in the current study, was organogenesis during the embryo stage of Atlantic salmon. Differences were noted in several *cell communication and signalling pathways*. Information regarding organogenesis in the context of domesticated and wild fish is limited. However, alteration of tissue development has been suggested for organs that arise from the neural crest, as a result of vertebrate domestication (Wilkins et al., 2014). Moreover, for the strains studied here, marginally earlier hatching time was reported for wild fish, (Solberg et al., 2014) that could indicate a slight shift in the rate of development of the different stocks.

The major biological functions perturbed in response to stress were common between stocks. Down-regulated pathways were mainly involved in *cellular processes,* including *cell cycle and meiosis*, and *genetic information processing*, such as *replication and repair*, *transcription* and *translation*. These changes are consistent with the reallocation of energy, away from growth and towards the restoration of a homeostatic state. Some *metabolic pathways*, mainly covering *energy*, *lipid* and *carbohydrate metabolism* were found to be up-regulated in response to stress, possibly reflecting energy mobilization in order to cover the energy demand of dealing with the stressor. *Digestive* and *endocrine system* related pathways were also up-regulated in response to stress, however, these functions were represented by a larger number of pathways in the wild pure and D♀W♂ crosses, than in the pure domesticated or W♀D♂ hybrid stocks, indicating a more substantial and/or consistent stress response of the former experimental groups. Stress response did not seem to be purely additive, since in addition to the biological functions affected in the pure crosses, further pathways were perturbed in the hybrids. Interestingly, these belonged to biological functions that were

identified as differentially expressed between wild and domesticated fish and were not particularly consistent between reciprocal hybrids. Down-regulation of *signal transduction* pathways and *nervous system* related pathways in response to stress were characteristics of W♀D♂ and D♀W♂ hybrids respectively.

7.2 Heritability of the identified transcriptomic differences

The main modes of inheritance of the genes differentially expressed between stocks were additivity and maternal dominance, contributing by 31-59% and 19-45% across life stages respectively. Although the latter ranged approximately from 20% to 30% in the majority of the life stages, and only increased to 45% in the sac fry. Consistently, most quantitative phenotypic traits of domesticated salmon have an additive basis. Maternal effects are also known to be common in salmonids, and are believed to be strongest in the juvenile stage and to decrease over time. Additivity was consistently least pronounced in the embryo stage, where maternal over-dominance accounted for 23-26% of the inheritance of the transcriptomic differences, whereas it was most pronounced in the 5 week post first-feeding fry. Domesticated traits governed by additivity are expected to weaken through repeated backcrossing with wild individuals. However, with the escapee issue remaining unresolved for decades and with no immediate solution in sight, large scale and regular escapes may continue to dilute locally adapted traits, even if they are additive. In addition, considering that it is domesticated salmon dams, rather than sires that are more likely to reproduce in nature (Fleming et al., 2000), the strong maternal effects detected are of particular concern. Furthermore, gene expression differences between backcross hybrids and wild fish have been reported to be equivalent, or more substantial than those between wild and domesticated fish (Roberge et al., 2008), and substantial (23-44%) over-dominant gene expression patterns have been reported for wild-domesticated Atlantic salmon backcross hybrids (Normandeau et al., 2009), suggesting that the genetic interaction between wild and domesticated Atlantic salmon may generate unpredictable phenotypes. Immune system related genes that were differentially

expressed between stocks, were mainly intermediate in the hybrids, although some of them showed maternal dominance, but only for the D♀W♂ hybrid cross, indicative of potential maternal environmental effects associated with aquaculture. This highly adaptive biological function has been consistently down regulated across life stages in the domesticated compared to wild fish. Considering the potential for increased pathogen/parasite load in some intensive fish farming activities, the disruption of locally adapted traits, especially ones associated with immune response may have detrimental consequences for wild populations.

7.3 Conclusions

The work reported in this thesis has demonstrated that biological functions affected by domestication include those associated with allocation of resources, involve reduction of information acquisition and processing systems and may lead to loss of local adaptation to wild conditions. Since such changes may affect key systems such as immunity, they can potentially have serious negative consequences under natural conditions. Transcriptomic differences observed between wild and domesticated stocks primarily exhibited additive and maternal dominant inheritance modes. Since gene-flow from farmed fish can be frequent and primarily concerns farmed females, this suggests that introgression due to repeated large scale escape events has the capacity to significantly erode local adaptation.

7.4 Limitations and future directions

It is important to note that the results presented here are based on whole individuals and single domesticated and wild stocks. To draw more general conclusions regarding the effects of domestication, multiple wild and domesticated strains would need to be examined. The logistics of conducting wider studies, are not, however, trivial. The resources required to conduct common-garden studies involving multiple wild and farmed stocks are not easily assembled and maintained. This is particularly the case given current legislation regarding handling, transport and biosecurity issues

associated with fish, especially wild stocks. The results of the presented work have highlighted the importance of using a large number of families and reciprocal hybrids to minimize individual parental effects and to allow the study of maternal effects respectively. It has been demonstrated that the effects of domestication vary across the course of development. Thus the study of additional developmental stages may prove beneficial. Employing backcross hybrids and common garden approaches under simulated natural conditions could help to shed light upon the adaptive or maladaptive status of domesticated phenotypes and underlying genotypes.

The presented results are based on a large number of probes designed for expressed sequence tags, available at the time of microarray design. Due to the analytical approach and techniques employed, it was only possible to measure the expression of polyadenylated RNA and the closed technology used gave a restricted set of potentially measurable transcripts. The decreasing price of next generation sequencing, which represents an open technology and which can allow both more direct quantification and access to a wider variety of RNA species, promises to provide additional power to resolve genomic differences by allowing investigation of the complete transcriptome in future studies.

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Appendix

Chapter 3

ProbeName	Primer Name	Primer sequence	Design	Product (bp)	Accession
Ssa#S35566877	SsaPoly10_1F	TATTGTGCAGCGTTG TAAAAACATTG	PrimerSelect	164	EG853255
	SsaPoly10_1R	ACCCCCTAAAAGTCA TGGTGTAAATC			
Ssa#KSS1847	Pescadillo_4F	TTGGTCCCATTGAAT ATGATGAGTGT	PrimerSelect	119	BT044790.1
	Pescadillo_4R	CAAAGTCACCTTGCG ATGGTTG			
Ssa#S35679411	MHCII_1F	CAGCGGAATATGTTC AGTAAATGGTG	PrimerSelect	160	EG914129
	MHCII_1R	TGAAGGAGGTACTTT CCAGAGGTGAT			
Ssa#STIR44213	EPHX_4F	GCTGCCTTCGAGGA GCCACAG	PrimerSelect	139	BT049657.2
	EPHX_4R	AGCCCTGCTGGTTTT TCTTCC			
Ssa#S30295328	MT28S_3F	GCCACTAGCGGCCT CTATCA	PrimerSelect	112	DW582292
	MT28S_3R	CCAGAATAATTTATG GAGGGATACAC			
N/A	EF1Aa	CCCCTCCAGGACGTT TACAAA	Solberg *et al.* 2012	57	AF321836
		CACACGGCCCACAG GTACA			
N/A	IGF-1	GTGTGCGGAGAGAG AGGCTTT	Solberg *et al.* 2012	68	M81904
		TGTGACCGCCGTGA ACTG			

Table 3.1 Details of primers used for RT-qPCR; EF1A and MT28S have been used as reference genes.

		Targets					References	
		MHCII	EPHX	IGF	Pesc	Poly10	EF1A	Mt28S
Sac fry	Wild	22.16	26.44	26.70	24.17	27.97	21.03	24.86
		22.45	26.76	26.72	24.20	27.81	20.82	24.88
		21.99	26.40	26.55	24.01	27.67	20.71	24.64
		22.82	26.58	26.77	24.08	28.12	20.86	24.84
		22.49	26.51	26.83	24.23	27.90	20.85	24.84
		22.26	26.58	26.72	24.18	28.33	20.92	24.95
	Hybrid	22.73	26.30	26.90	24.23	28.38	20.85	24.98
		22.82	26.25	26.68	24.10	28.60	20.77	24.90
		22.58	26.24	26.72	24.10	28.66	20.89	24.94
		22.49	26.29	26.73	24.12	28.18	21.00	24.91
		22.21	26.36	26.69	24.12	28.25	20.76	24.78
		22.87	26.28	26.54	24.08	28.19	20.82	24.79
	Domesticated	22.92	26.17	26.84	24.02	29.36	20.73	24.89
		23.17	26.34	26.92	24.29	29.49	20.91	25.01
		22.93	26.16	26.85	24.11	29.57	20.86	24.94
		23.07	26.32	26.90	24.19	29.57	20.89	24.96
		23.00	26.16	27.04	24.08	29.40	20.88	24.88
		22.78	26.19	26.83	24.19	28.65	20.81	24.82
Feeding fry	Wild	21.19	25.47	26.73	24.34	28.18	20.65	25.02
		21.34	25.53	26.91	23.89	27.83	20.45	24.72
		20.96	25.47	26.67	24.00	27.77	20.56	24.78
		20.81	25.28	26.80	24.17	27.97	20.52	24.75
		20.97	25.30	26.73	24.16	27.99	20.56	24.92
		21.28	25.31	26.69	24.38	27.51	20.70	24.91
	Hybrid	21.49	25.36	26.87	24.58	28.71	20.77	25.01
		21.50	25.29	27.02	24.41	28.36	20.74	25.04
		21.71	25.25	26.89	24.37	28.38	20.76	24.98
		21.51	25.31	26.82	24.49	28.33	20.83	25.05
		21.63	25.24	26.67	24.43	28.31	20.80	25.03
		21.66	25.37	26.86	24.51	28.64	20.74	24.99
	Domesticated	22.23	25.18	26.76	24.54	28.89	20.65	24.97
		22.15	25.29	26.85	24.61	28.84	20.80	25.03
		21.95	25.25	26.86	24.63	28.68	20.77	25.06
		22.08	25.26	26.78	24.29	28.68	20.68	24.90
		22.53	25.24	26.94	24.60	28.85	20.81	24.99
		22.42	25.17	26.52	24.33	28.78	20.73	24.94

Table 3.2 CT values for RT-qPCR

Sac fry

	Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
Domesticated	MHCII	TRG	0.96	0.675	0.561 - 0.825	0.534 - 0.966	0.003	DOWN
	EPHX	TRG	0.97	1.271	1.183 - 1.392	1.083 - 1.533	0.002	UP
	IGF	TRG	1	0.901	0.844 - 0.970	0.771 - 1.014	0.008	DOWN
	Pesc	TRG	0.97	1.02	0.958 - 1.092	0.910 - 1.130	0.447	
	Poly10	TRG	0.87	0.432	0.364 - 0.530	0.348 - 0.709	0.001	DOWN
	EF1A	REF	0.85	1.033				
	Mt28S	REF	0.94	0.968				
	Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
Hybrid	MHCII	TRG	0.96	0.852	0.659 - 1.088	0.596 - 1.347	0.145	
	EPHX	TRG	0.97	1.204	1.088 - 1.307	1.012 - 1.470	0.003	UP
	IGF	TRG	1	1.014	0.937 - 1.099	0.871 - 1.154	0.683	
	Pesc	TRG	0.97	1.025	0.965 - 1.092	0.927 - 1.144	0.353	
	Poly10	TRG	0.87	0.782	0.641 - 0.913	0.611 - 1.039	0.01	DOWN
	EF1A	REF	0.85	1.021				
	Mt28S	REF	0.94	0.979				

Feeding fry

	Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
Domesticated	MHCII	TRG	0.96	0.512	0.419 - 0.614	0.383 - 0.729	0.001	DOWN
	EPHX	TRG	0.97	1.227	1.118 - 1.364	1.055 - 1.494	0.001	UP
	IGF	TRG	1	1.077	0.956 - 1.239	0.898 - 1.399	0.263	
	Pesc	TRG	0.97	0.871	0.768 - 0.971	0.747 - 1.053	0.024	DOWN
	Poly10	TRG	0.87	0.621	0.531 - 0.701	0.455 - 0.768	0.002	DOWN
	EF1A	REF	0.85	0.992				
	Mt28S	REF	0.94	1.008				
	Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
Hybrid	MHCII	TRG	0.96	0.807	0.699 - 0.934	0.651 - 1.101	0.017	DOWN
	EPHX	TRG	0.97	1.195	1.088 - 1.346	1.018 - 1.461	0.004	UP
	IGF	TRG	1	1.049	0.920 - 1.206	0.846 - 1.350	0.456	
	Pesc	TRG	0.97	0.912	0.845 - 1.002	0.775 - 1.039	0.058	
	Poly10	TRG	0.87	0.782	0.652 - 0.919	0.511 - 0.970	0	DOWN
	EF1A	REF	0.85	0.994				
	Mt28S	REF	0.94	1.006				

Table 3.3 RT-qPCR results according to REST

Chapter 4

Name	Functional group	p-value_-log10	fold change_log2
CCL3	Signal transduction	4.689488152	-1.6971343
PARD3	Signal transduction	1.800520402	-1.5978892
CCL19	Signal transduction	1.312347929	1.8000811
NFKBIA	Signal transduction	1.457699628	-1.0252575
MAP2K4	Signal transduction	1.399606387	1.284033
PDGFRA	Signal transduction	3.57736048	0.793015
PPP1C	Signal transduction	2.778185587	-1.1623192
CTNNB1	Signal transduction	1.427892371	1.5622821
BIRC2_3	Signal transduction	1.317854066	0.7432575
CDHE	Signal transduction	1.656005061	1.0403646
TNFSF14	Signal transduction	1.526118826	-1.9623429
TNFRSF3	Signal transduction	1.421252058	1.6956582
CAMK2	Signal transduction	1.578350968	-1.1090248
CREBBP	Signal transduction	2.257954053	1.4059732
DLG2_3	Signal transduction	3.778249594	1.1656635
APC	Signal transduction	2.705333427	-0.8242481
SKP1	Signal transduction	2.648103438	0.9079287
BAMBI	Signal transduction	1.907878203	0.86884815
SMAD4	Signal transduction	1.823576777	0.83845985
NR4A1	Signal transduction	1.717222887	0.83774984
NTRK2	Signal transduction	1.569566896	1.5780388
ADCY2	Immune system	1.305110278	0.91063076
PIK3C	Immune system	4.365008129	-1.0077442
FYN	Immune system	1.759791457	-2.0443883
PAK7	Immune system	2.773139133	-0.8022565
VAV	Immune system	1.744046597	-1.2292999
IFNA	Immune system	2.075134347	-2.0615487
CCLX	Immune system	1.387554919	-1.103246
CXCL9	Immune system	6.469407206	-1.3250206
PRF1	Immune system	3.882330662	1.7206397
PREX1	Immune system	1.59034368	1.1014411
COL1AS	Cell communication	1.921221785	-1.682397
ITPR2	Cell communication	2.822320019	-1.9511616
PPP1R12A	Cell communication	1.513581126	0.7566116
TUBA	Cell communication	2.375037431	1.8463984
TLN	Cell communication	1.353219425	-0.89632654
TNFRSF11B	Signaling molecules and interaction	1.462552341	-2.057237
AGRN	Signaling molecules and interaction	1.425871959	-1.6677104
TRPC1	Digestive system	1.480239977	0.94898015
SLC26A6	Digestive system	2.426008597	1.716381
SLC12A2	Digestive system	1.846289318	-1.9177654
RAB8A	Digestive system	1.6977604	0.67278224
TPCN2	Digestive system	1.543792726	-0.7969878
CLCN2	Digestive system	1.412004727	0.6681599

CALCA	Circulatory system	3.661209109	-3.6516645
EIF5	Translation	4.686538486	1.0630834
RPL7	Translation	3.415837776	0.9076543
RPL6	Translation	2.699879217	1.3333608
RPL22	Translation	1.935584057	0.7329193
EIF3E	Translation	1.419468758	1.4391894
PFK	Carbohydrate metabolism	8.52381373	1.8962305
MGAM	Carbohydrate metabolism	2.732741093	0.81598234
GALT	Carbohydrate metabolism	2.709645932	0.8037558
B3GNT4	Glycan biosynthesis and metabolism	2.458650431	1.7029111
FUT9	Glycan biosynthesis and metabolism	2.024973814	0.9678975
B3GALT1	Glycan biosynthesis and metabolism	1.57556845	0.812376
SIAT6	Glycan biosynthesis and metabolism	1.533210797	-1.1541834
ALT	Energy metabolism	2.704668186	3.0938559

Table 4.1 Details of the plotted essGenes

	p.geomean	stat.mean	p.val	q.val	set. size	MM.5	MM.4	MM.1	MM.6	MM.2	MM.3
ko04060 Cytokine-cytokine receptor interaction	0.012711	2.193731	5.34E-08	9.66E-06	86	0.027592	0.016491	0.145794	0.008283	0.007229	0.001062
ko04720 Long-term potentiation	0.030715	1.847479	5.11E-06	0.000462	28	0.026101	0.019585	0.020534	0.099254	0.003875	0.207978
ko04080 Neuroactive ligand-receptor interaction	0.035926	1.773812	7.73E-06	0.000466	116	0.091397	0.052628	0.015286	0.02008	0.124332	0.011712
ko04660 T cell receptor signaling pathway	0.043881	1.705848	1.74E-05	0.000789	58	0.047963	0.090464	0.036527	0.096989	0.019008	0.024435
ko04650 Natural killer cell mediated cytotoxicity	0.047368	1.642301	3.82E-05	0.001381	39	0.017952	0.192854	0.166324	0.013125	0.037064	0.040326
ko04912 GnRH signaling pathway	0.066405	1.49714	0.000144	0.004297	43	0.108749	0.05971	0.035754	0.113798	0.024622	0.131811
ko04062 Chemokine signaling pathway	0.069682	1.470252	0.00017	0.004297	85	0.077992	0.042495	0.049691	0.040581	0.123887	0.138268
ko04380 Osteoclast differentiation	0.068676	1.461182	0.00019	0.004297	67	0.071618	0.20827	0.062578	0.102712	0.070439	0.015535
ko04064 NF-kappa B signaling pathway	0.064502	1.451147	0.000215	0.004321	63	0.222151	0.075555	0.06296	0.052265	0.230133	0.005666
ko04664 Fc epsilon RI signaling pathway	0.071254	1.445665	0.000256	0.004642	29	0.146841	0.127972	0.035368	0.011652	0.110288	0.153233
ko04270 Vascular smooth muscle contraction	0.066974	1.411909	0.000315	0.00519	56	0.034247	0.003893	0.122952	0.136285	0.136229	0.296508
ko04540 Gap junction	0.079111	1.397714	0.000356	0.005363	40	0.094872	0.034301	0.192664	0.042449	0.057124	0.161246
ko04390 Hippo signaling pathway	0.081888	1.375651	0.000403	0.005617	73	0.088532	0.200295	0.105361	0.065853	0.025856	0.094781
ko04510 Focal adhesion	0.079949	1.347962	0.000508	0.006567	99	0.272229	0.031316	0.030072	0.033095	0.163039	0.18878
ko04010 MAPK signaling pathway	0.095475	1.265334	0.001008	0.012159	115	0.130648	0.043502	0.272337	0.174643	0.103541	0.027063
ko04970 Salivary secretion	0.092811	1.263228	0.001171	0.01325	32	0.242558	0.015031	0.073624	0.063949	0.101839	0.365631
ko04514 Cell adhesion molecules (CAMs)	0.093446	1.226588	0.001441	0.015343	64	0.310126	0.019667	0.176541	0.050987	0.03316	0.365735
ko04724 Glutamatergic synapse	0.103596	1.218177	0.001541	0.015499	50	0.193778	0.040859	0.109555	0.341839	0.039712	0.104977
ko04972 Pancreatic secretion	0.110603	1.210111	0.001645	0.01567	43	0.071174	0.058559	0.116591	0.090094	0.262087	0.159543
ko04310 Wnt signaling pathway	0.113355	1.173489	0.002128	0.01926	68	0.191634	0.051671	0.305096	0.045587	0.10174	0.151413
ko04630 Jak-STAT signaling pathway	0.122962	1.157096	0.002431	0.020949	51	0.126478	0.162435	0.15232	0.080148	0.184884	0.074538
ko04620 Toll-like receptor signaling pathway	0.127491	1.132952	0.002917	0.024	48	0.095796	0.172894	0.094037	0.241735	0.108561	0.105062
ko04916 Melanogenesis	0.12039	1.1241	0.003211	0.025269	41	0.220473	0.066457	0.375724	0.031605	0.116391	0.150351
ko04918 Thyroid hormone synthesis	0.132612	1.10373	0.003697	0.027878	35	0.233164	0.057868	0.095259	0.157711	0.131779	0.2036
ko00534 Glycosaminoglycan biosynthesis - heparan sulfate / heparin	0.124786	1.101493	0.004662	0.033756	13	0.134519	0.03641	0.534011	0.063663	0.228328	0.099311
ko04330 Notch signaling pathway	0.140147	1.072156	0.004887	0.03399	21	0.181902	0.107461	0.048656	0.199336	0.174382	0.229183

ko04662 B cell receptor signaling pathway	0.131627	1.060499	0.00507	0.03399	38	0.407232	0.270897	0.201366	0.046897	0.078854	0.063309
ko04668 TNF signaling pathway	0.141944	1.049439	0.005271	0.034071	62	0.146754	0.270602	0.068429	0.196804	0.208891	0.073214
ko04976 Bile secretion	0.139979	1.029627	0.006212	0.038006	41	0.081444	0.047887	0.101268	0.180419	0.417026	0.253157
ko04730 Long-term depression	0.134815	1.036547	0.006299	0.038006	25	0.453692	0.046839	0.165067	0.239114	0.034755	0.205952
ko04020 Calcium signaling pathway	0.147585	0.993069	0.007745	0.045131	71	0.409307	0.054485	0.117469	0.10744	0.114694	0.320106
ko04913 Ovarian steroidogenesis	0.155299	0.995034	0.007979	0.045131	28	0.151482	0.163568	0.064962	0.16202	0.365554	0.147154
ko04915 Estrogen signaling pathway	0.149161	0.986861	0.008258	0.045295	41	0.106894	0.062743	0.227044	0.209664	0.075063	0.459573
ko04151 PI3K-Akt signaling pathway	0.161924	0.949848	0.010119	0.053868	147	0.099901	0.142294	0.352839	0.065835	0.21035	0.259496
ko00601 Glycosphingolipid biosynthesis - lacto and neolacto series	0.145307	0.924551	0.014362	0.073513	15	0.063314	0.196863	0.401712	0.033591	0.691918	0.080884
ko04725 Cholinergic synapse	0.177171	0.895908	0.014621	0.073513	44	0.096566	0.241642	0.124741	0.218883	0.118359	0.410147
ko04391 Hippo signaling pathway – fly	0.177816	0.876597	0.016811	0.082235	32	0.15716	0.321659	0.119143	0.414685	0.055727	0.227105
ko04520 Adherens junction	0.186153	0.858291	0.018411	0.085323	43	0.276164	0.314046	0.068518	0.158026	0.330704	0.133996
ko04360 Axon guidance	0.186811	0.855635	0.018513	0.085323	56	0.158765	0.239715	0.460692	0.130042	0.180309	0.103384
ko00514 Other types of O-glycan biosynthesis	0.180645	0.867608	0.018856	0.085323	16	0.104401	0.063226	0.369683	0.13931	0.431653	0.236816
ko00500 Starch and sucrose metabolism	0.187797	0.848288	0.02022	0.089265	22	0.134518	0.076247	0.242716	0.146485	0.466204	0.258025

Table 4.2 Details of all significant 2d pathways (q (corrected p)<0.1)

Chapter 5

KO	Annotation	Stress WD	Stress DW	Control WD	Control DW
K08202	MFS transporter, OCT family, solute carrier family 22 (organic cation transporter), member 4/5	Wild dominant	Additive	#N/A	#N/A
K01025	sulfotransferase family 1, cytosolic sulfotransferase 3	Wild dominant	Additive	#N/A	#N/A
K10408	dynein heavy chain, axonemal	Wild dominant	Additive	#N/A	#N/A
K17989	L-serine/L-threonine ammonia-lyase	Wild dominant	Additive	#N/A	#N/A
K01823	isopentenyl-diphosphate delta-isomerase	Wild dominant	Additive	#N/A	#N/A
K01897	long-chain acyl-CoA synthetase	Wild dominant	Additive	#N/A	#N/A
K17963	peroxisome proliferator-activated receptor gamma coactivator-related protein 1	Wild dominant	Additive	Wild dominant	Additive
K01493	dCMP deaminase	Wild dominant	Additive	#N/A	#N/A
K00252	glutaryl-CoA dehydrogenase	Wild dominant	Additive	#N/A	#N/A
K00025	malate dehydrogenase	Wild dominant	Additive	#N/A	#N/A
K17854	cytochrome P450, family 2, subfamily K	Wild dominant	Additive	#N/A	#N/A
K00286	pyrroline-5-carboxylate reductase	Wild dominant	Additive	#N/A	#N/A
K03998	complement component 8 subunit beta	Wild dominant	Additive	#N/A	#N/A
K07424	cytochrome P450, family 3, subfamily A	Wild dominant	Additive	#N/A	#N/A
K02726	20S proteasome subunit alpha 2	Wild dominant	Additive	#N/A	#N/A
K10808	ribonucleoside-diphosphate reductase subunit M2	Wild dominant	Additive	#N/A	#N/A
K10090	galectin-2	Wild dominant	Additive	Wild dominant	Additive
K00489	cholesterol 7alpha-monooxygenase	Wild dominant	Additive	#N/A	#N/A
K01330	complement component 1, r subcomponent	Wild dominant	Additive	#N/A	#N/A
K17771	mitochondrial import receptor subunit TOM7	Wild dominant	Additive	Wild dominant	Domesticated dominant
K13279	peroxiredoxin 1	Wild dominant	Additive	#N/A	#N/A
K09542	crystallin, alpha B	Wild dominant	Additive	#N/A	#N/A
K08750	fatty acid-binding protein 1, liver	Wild dominant	Additive	Wild dominant	Additive
K09633	transmembrane protease, serine 2	Wild dominant	Additive	Wild dominant	Domesticated dominant
K00157	aldehyde oxidase	Wild dominant	Additive	#N/A	#N/A
K15013	long-chain-fatty-acid--CoA ligase ACSBG	Wild dominant	Additive	#N/A	#N/A
K10205	elongation of very long chain fatty acids protein 2	Wild dominant	Additive	#N/A	#N/A
K17275	plastin-1	Wild dominant	Additive	#N/A	#N/A
K10372	troponin T, slow skeletal muscle	Wild dominant	Additive	Wild dominant	Domesticated dominant
K13646	lysyl hydroxylase/galactosyltransferase/glucosyltransferase	Wild dominant	Additive	Wild dominant	Domesticated dominant
K10359	myosin VII	Wild dominant	Wild dominant	#N/A	#N/A
K01507	inorganic pyrophosphatase	Wild dominant	Wild dominant	#N/A	#N/A
K05995	dipeptidase E	Wild dominant	Wild dominant	#N/A	#N/A
K05854	tyrosine-protein kinase Lyn	Wild dominant	Wild dominant	#N/A	#N/A

Table 5.1 Details of the genes plotted for wild domesticated effect