Maternal effects and phenotypic mismatch in hatchery-reared Atlantic salmon.

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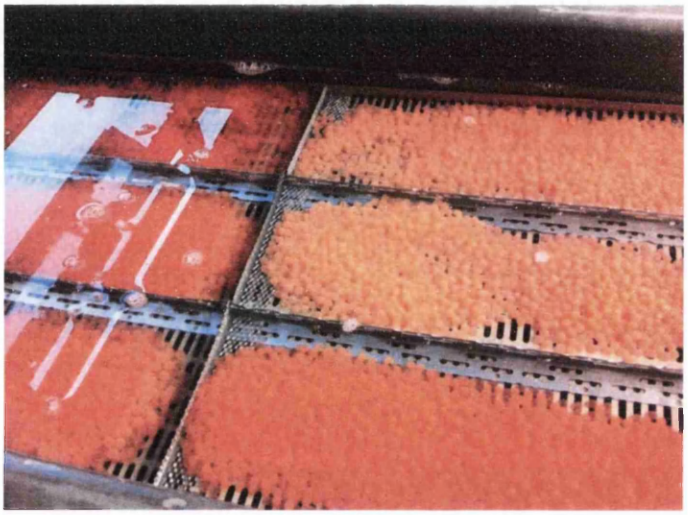

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ABSTRACT

Phenotypic variation was previously thought to be the result of complex interactions between an individual's genotype and the environment in which it exists. It is, however, now evident that an individual's phenotype may also be shaped by the environmental variation experienced by the mother, i.e. maternal effects. Environmental maternal effects have the potential to generate rapid phenotypic change in a population and so may be particularly important for evolution at ecological time-scales. The general aim of this thesis was to examine how maternal effects may influence offspring fitness and life history traits in Atlantic salmon (*Salmo salar* L.1758). For this species, the early juvenile period is the most critical due to their complex life cycle. Offspring rely on maternal provisioning during the early stages of development for growth and survival. Several studies on Atlantic salmon have emphasised the benefits of developing from larger eggs, yet it is unclear how the effects of rearing environment influence early life development. The thesis therefore investigated the effects of variation in maternal provisioning and female rearing environment on the development and physiology of embryos, the behaviour of newly emerged fry and the survival of fry released into the wild. Also assessed were the phenotypic changes among juvenile salmon released into the wild compared to those retained in the hatchery. For this maternal provisioning was manipulated by varying the length of time mothers from the same genetic background were maintained in captivity (2 months, 14 months and 26 months). The results of this thesis demonstrate that both maternal provisioning and female rearing environment alter the development and behaviour of salmon fry, opercular beat rate (a proxy for metabolic rate) and yolk sac absorption, and ultimately survival in the wild. Hatchery-reared fry were found to be maladapted to the natural environment for a number of phenotypic traits which are known to impact survival and the longer fry are retained in the hatchery prior to release the more phenotypically mismatched to the natural environment they become. However, increased egg size brought about my retaining females in captivity improved survival.

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CONTENTS

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INTRODUCTION

Phenotypic variation provides the raw material for natural selection and understanding the causes and consequences of this variation is the target of evolutionary ecology (Pianka, 2011). One of Darwin's greatest insights was to realise that phenotypic variation provides an opportunity for natural selection to modify the distribution of fitness relevant traits, thereby increasing the frequency of characteristics that enhance the probability of an organism surviving and reproducing (Darwin, 1859).

It was previously considered in studies of evolutionary ecology that phenotypic variation was mainly the result of complex interactions between an individual's genotype and the environment it inhabited, yet it is now evident that an individual's phenotype may also be shaped by maternal effects (Bernardo, 1996a). Thus, the environmental variation (e.g. temperature, nutritional availability, and photoperiod) experienced by the mother can be translated into phenotypic variation in the offspring (Mousseau and Fox, 1998a) and these inherited environmental effects are of broad significance for phenotype evolution (Reinhold, 2002). By definition, maternal effects are the contribution of the maternal parent to the phenotype of its offspring beyond equal chromosomal contribution expected from each parent (Roach and Wulff, 1987; Mousseau and Fox, 1998a). In particular, the influences the maternal environment has on her offspring's phenotype are known as environmental maternal effects, and reflect transgenerational responses to past and current environment experienced by the mother (Duckworth, 2009). Mothers that are exposed to altered environments may also be subjected to epigenetic changes (changes in gene expression that do not involve changes in DNA sequences) (Richards *et al.*, 2010). Epigenetic effects can be transgenerational, i.e. inherited over a number of generations. For example, elevated temperatures experienced by female sheepshead minnow (*Cyprinodon variegatus*) prior to fertilization can modify offspring thermal reaction norms for growth (Salinas and Munch, 2012). These epigenetic effects are potentially important for offspring development and may compensate for a poor start under some conditions (Jonsson and Jonsson, 2014).

Environmentally induced maternal effects can exert indirect genetic effects on offspring and affect development, behaviour and ultimately survival (Wolf *et al.*,

1998; Todd *et al.*, 2011). In many species, mothers can alter offspring development, by manipulating the size and quality of the eggs, and by controlling where, when, and how those eggs are placed (Mousseau and Fox, 1998b). For example, when female keelback snakes (*Tropodonophis mairii*) are presented with a choice of nesting sites, mothers often select moist substrates, which significantly increases offspring body size at hatching (Brown and Shine, 2004). Mothers can also alter the timing of development of their offspring; for example, in many insects females which are experiencing deteriorating environmental conditions tend to produce a high proportion of diapausing (suspended development) offspring (Mousseau and Fox, 1998a) in order to maximise the survival of the next generation (Hairston Jr and Olds, 1984).

Why study maternal effects?

Evolutionary biology aims to gain an understanding of how variation is maintained between individuals and populations (Stearns, 1992), and maternal effects were once considered to be a nuisance parameter, which inflates estimates of the genetic basis of adaptive traits, and as such responses to selection (Räsänen and Kruuk, 2007; Wolf and Wade, 2009). However, maternal effect are now recognised in evolutionary ecology as one of the most important influences on offspring phenotype (Mousseau and Fox, 1998a; Marshall and Uller, 2007; Green, 2008). Most of the environmental variables known to contribute to environmental maternal effects are permanent components of species' environments (e.g. population density, seasonal features, food and habitat quality) highlighting their importance in ecological and evolutionary processes (Rossiter, 1996). Therefore, maternal effects often have evolutionary consequences for populations. This could occur either through genetic change in response to natural selection, potentially driving rapid between-population divergence or through an increase in phenotypic plasticity, which would facilitate population persistence under changing environments (Räsänen and Kruuk, 2007).

The development of more powerful experimental designs and genetic models in recent years has enabled maternal effects to be estimated independently from other genetic and non-genetic influences (Maria *et al.*, 1993; Robinson, 1996; McAdam *et al.*, 2002). Therefore consideration of maternal effects has been increasingly incorporated into evolutionary studies, including studies of evolutionary ecology and

conservation biology due to their importance in facilitating evolutionary change (Bernardo, 1996a; Mousseau *et al.*, 2009). Maternal effects are capable of influencing every target of natural selection in a wide variety of organisms, and many maternal effects appear to have been shaped by selection as an adaptive response to heterogeneous environments (Mousseau *et al.*, 2009).

Effect of maternal provisioning on offspring quality

The environment can play an important role in maternal nutrition, for example, local resource availability can affect a female's condition, energy reserves and maintenance demands (Bernardo, 1996b). This can in turn influence the amount of reserves allocated to reproduction with effects on number, size and quality of offspring produced (Glazier, 1992; Bernardo, 1996a). Maternal influences on offspring size and quality have been widely studied due to their direct consequences for fitness and life history traits (Mousseau and Fox, 1998b); such effects are particularly evident in species without parental care as it is the only opportunity for parents to invest into the care of their offspring (Rossiter *et al.*, 1993).

For oviparous species, egg size is a good proxy for offspring quality (Krist, 2011; Régnier *et al.*, 2013). Any increase in egg provisioning, however, is costly for mothers and a trade-off often exists between egg size and egg number (Stearns, 1992). A number of optimality models of this trade-off have been developed in an effort to identify the selective forces shaping egg size evolution, the first and perhaps best known being that by Smith and Fretwell (1974). This model postulates that there is a parent - offspring conflict, where fitness of the offspring increases at a diminishing rate with resource investment, but the fitness of the maternal parent has an optimum (Smith and Fretwell, 1974). Models of egg size evolution consider that the energy available for reproduction is limited at any given time, and that offspring fitness increases with investment per offspring (Fox and Czesak, 2000; Krist, 2011). Nutritional and physical limitations such as female size, food availability and diet may constrain the amount of resources invested into offspring, giving rise to high variability in egg size within a species (Christians, 2002). Furthermore, optimal egg size may be expected to be context-dependent, as producing large embryos should be more beneficial under harsh competitive environments (Einum and Fleming, 1999)

and mothers may be able to predict the environment of their future offspring and allocate resources appropriately (Crean and Marshall, 2009).

In addition to between female variation in egg size, variation in allocation of resources within a clutch has also been noted in a number of species, and is thought to represent an adaptive response by females living in fluctuating and difficult to predict environments (Koops *et al.*, 2003). This strategy is known as 'bet-hedging' and is thought to ensure that at least a few offspring are of optimal size for any given environment, hence ensuring some reproductive success under most conditions (Marshall *et al.*, 2008).

Fish as models for examining maternal effects

Fish are arguably the most diverse group of vertebrates in terms of morphology, physiology, life histories and environmental conditions they experience (Heath and Blouw, 1998). Fish comprise more than 30,000 species (FishBase, 2011, IUCN Red List 2007) and few aquatic ecosystems exist that have not been colonized by at least some fish (Crollius and Weissenbach, 2005). Fish, therefore, provide good models for studying adaptive responses to a wide range of natural and anthropogenic environmental conditions (Cossins and Crawford, 2005). Maternal effects have been studied in a number of marine (Pepin, 1991; McCormick, 2003; Berkeley *et al.*, 2004), freshwater (Taborsky *et al.*, 2007; Venturelli *et al.*, 2010) and migratory fishes, e.g. salmonids (Einum and Fleming, 1999; Heath *et al.*, 1999). Typically, studies of maternal effects on fish have been carried out in species with small body size that reproduce quickly and are easy to rear in the laboratory (Cossins and Crawford, 2005). In contrast, studying the adaptive responses of fish in the wild is difficult, as the ability to manipulate phenotypic variation and control for environmental fluctuations is limited (Endler, 1986).

Salmonids are a particularly useful study species as they are widely cultured in hatcheries for conservation purposes (Fraser, 2008) which allows for the comparison of cultured and wild populations to be made. They also show considerable adaptive variation, defined as heritable phenotypic variation that is sorted by natural selection into different environmental niches, so enhancing fitness in specific environments (Garcia de Leaniz *et al.*, 2007a). Salmonids are good model

organisms for testing theories of egg size evolution, as they provide little post-partum care to offspring, such that egg size is a good proxy for the amount of energy invested per offspring (Burton *et al.*, 2013a). Given the availability of a large number of offspring and the possibility to manipulate the rearing environment of both the broodstock and the offspring, salmonids are ideal models for studying maternal effects.

Maladaptation of hatchery-reared salmonids

The rearing of salmonids in captivity is used for the restoration, conservation and enhancement of wild populations (Blanchet *et al.*, 2008). There are, however, a number of concerns regarding the stocking of genetically and phenotypically maladapted fish in the wild (Kostow, 2009). Unlike the natural environment, hatchery facilities provide predator-free environments with a plentiful supply of food which promotes fast growth (Fleming and Einum, 1997). Once released into the wild, hatchery fish typically perform poorly as there is often a phenotypic mismatch with the natural environment (Brown *et al.*, 2003). Captive breeding strategies are being developed that aim to preserve the genetic and phenotypic integrity of wild populations as much as possible (Duchesne and Bernatchez, 2002). For this, wild broodstock from the target river system are maintained in captivity for reproduction and their offspring are released at an early developmental stage to minimize the effects of rearing environment on phenotypes (Dannewitz *et al.*, 2004). There is, however, an added risk of inadvertent selection for domestic traits in artificial environments through maternal effects, as mothers which spend an increasing amount of time in captivity may alter their reproductive investment due to the reduced environmental variation (in relation to the wild environment) and excess food supply (Fraser, 2008). Ultimately, both the female's phenotype or environment can influence the phenotype of the offspring, and can have underlying consequences on juvenile behaviours and life-history traits which are important to fitness once released into the wild (Einum and Fleming, 2000b).

The individual differences in the way animals respond to stressful situations are often referred to as stress coping styles (Weiss, 1968), a term which is often used to describe suits of behavioural and physiological responses to challenges that are

constant over time (Koolhaas *et al.*, 1999). Such behavioural and physiological differences have been clustered into two characteristic responses, termed proactive and reactive stress coping styles (Koolhaas *et al.*, 1999; Vaz-Serrano *et al.*, 2011). Since most studies of behavioural traits associated with stress-coping styles have been done on individuals with social experience (Höglund *et al.*, 2008), studies on socially naive salmon alevins during yolk-sac absorption could provide information about the maternal influence on an individual's stress coping strategy.

Body shape and size is believed to be under strong selection in fish as it greatly affects performance, but the relation between body shape and fitness is likely to be a complex one. As morphology affects swimming efficiency, feeding ability and predator avoidance it is likely to be an adaptive trait (Pakkasmaa and Piironen, 2000; Drinan *et al.*, 2012). Confinement in hatchery tanks with low water velocity and plentiful food increases fat deposition and results in deepening of the body amongst hatchery–reared salmonids in comparison with fish in the wild (Pulcini *et al.*, 2013). This serves to highlight the different selective pressures that fish experience in natural and artificial environments (Lorenzen *et al.*, 2012).

Crypsis, defined as the ability to go unnoticed or stay hidden away from potential predators (Starrett, 1993), is another trait that may be affected by artificial rearing in hatcheries. It is an effective anti-predatory tactic used by many fish, including salmonids (Cox *et al.*, 2009). Salmonids show considerable plasticity in parr mark pigmentation which depends on diet, but also responds to a number of environmental variables including water transparency and substrate type, which is likely to be under selection (Culling *et al.*, 2013).

Fluctuating asymmetry has been widely used as a measure of developmental instability i.e. the inability by an embryo to produce a consistent phenotype in a given environment and thus has become the focus of considerable attention (Johnson *et al.*, 2004). The development of bilateral structures on opposites sides of an organism is controlled by the same genes, and any deviations from perfect bilateral symmetry are thought to result from environmental and genetic stressors (Johnson *et al.*, 2004). High levels of fluctuating asymmetry in some organisms (Vollestad and Hindar, 1997; Yurtseva *et al.*, 2010) have been linked to maternal effects as well as

environmental fluctuations during embryo development, and reduced genetic variation (Leary *et al.*, 1985a; Leary *et al.*, 1985b).

It is evident that maternal effects can affect a number of adaptive traits and that these traits can, in turn, have an important influence on the success of offspring in the wild. However, the extent to which the captive rearing environment experienced by mothers can affect offspring life history traits is largely unknown. Knowledge on the effects of female rearing environment on offspring fitness would therefore be useful to salmonid conservation programmes that rely on the reconditioning of broodstock to achieve multiple, repeated spawnings over consecutive years. Increasing the time mothers spend in captivity may result in a relaxation of a number of important traits, such as predator avoidance and foraging behaviour. Captive rearing could also weaken the strength of maternal effects and diminish the benefits that offspring may indirectly derive from the environment experienced by their mothers.

AIMS AND OBJECTIVES

The overall aim of this thesis was to investigate how maternal effects influence offspring fitness and life history traits using the migratory Atlantic salmon (*Salmo salar*) as a model species. To do this, maternal provisioning was manipulated by varying the length of time mothers from the same genetic background were maintained in captivity (2 months, 14 months and 26 months) and compared the phenotypic variation and early life history traits of the offspring. For this a split - brood breeding design was used so that two sires and three dams (one from each experimental group) were mated to produce 6 families in a 3 x 2 design (Fig. 1.1). Hatchery-reared fry were later released into the wild and compared with a control group kept in the hatchery to examine the responses of fish from different maternal background to environmental variation. It was hypothesised that captive rearing (characterised by a plentiful supply of food, environmental homogeneity and absence of predators) would increase maternal investment, which would consequently increase offspring metabolic rate and aggression and ultimately reduce adaptation in the wild.

Chapters outline

Chapter I. Maternal effects on embryo development in Atlantic salmon

A laboratory based study was undertaken to establish the effect of maternal rearing environment and provisioning on early life history traits. The study focused on the timing of critical events such as hatching, yolk sac absorption and emergence from the gravel of offspring derived from mothers which had spent varying lengths of time in a hatchery environment. The study asked the following question:

How does variation in maternal rearing environment affect offspring development?

It was expected that mothers retained in the hatchery would produce larger eggs for their body size, and embryos would hatch later and take longer to absorb their yolk sac reserves compared to controls from wild, maiden females. This is due to the expectation that development should be slower in larger than in smaller embryos, as a result of a decreased metabolic rate (Valdimarsson *et al.*, 2002).

Chapter II. Maternal effects on early behaviour in Atlantic salmon

The second chapter focused on variation in agonistic behaviour and ventilation rate (a proxy for metabolic rate), and their correlation with stress coping styles, of offspring derived from mothers which had spent varying lengths of time in a hatchery environment. Offspring were assessed for variation in agonistic behaviour at the time they emerged from simulated gravel nests, a critical period for survival and where life history strategies can have strong effects on fitness. The following question was asked:

How does variation in maternal rearing environment affect aggression and metabolic rate of recently emerged salmon fry?

It was expected that offspring from mothers retained in the hatchery would be more aggressive than those from control mothers due to an inadvertent selection for domesticated traits (Johnsson and Björnsson, 1994; Weber and Fausch, 2003). It was also expected that aggression would increase with an earlier timing of emergence and an increase in metabolic rate due to the underlying relation between behavioural and physiological traits associated with stress-coping styles (Andersson *et al.*, 2013).

Chapter III. Maladaptation and phenotypic mismatch in hatchery-reared Atlantic salmon released in the wild.

Hatchery-reared fish typically perform poorly in the wild but the reasons are not clear. In chapter three changes in body shape, fluctuating asymmetry (FA), and crypsis were compared among Atlantic salmon fry released into the wild and those kept in the hatchery. The questions asked were:

How long does it take for hatchery fish to adapt to the natural environment, and for how long do hatchery traits persist in the wild?

It was expected that fry released into the natural environment would become phenotypically similar to wild fish and more dissimilar to those retained in the hatchery. As captive rearing relaxes natural selection and therefore generates individuals with extreme phenotypes which are maladapted to the wild environment, natural selection and phenotypic plasticity will be expected to favour those phenotypes that increase fitness in local environments (Solem *et al.*, 2006).

Chapter IV. Maternal investment and juvenile survival in Atlantic salmon: a field test of the 'big old fat fecund female fish (bofff)' hypothesis

Fitness during early life can depend critically on the quality of maternal provisioning, which often translates into variation in egg size. The final chapter tested for the existence of genotype- by- environment interactions on phenotypic traits of juvenile Atlantic salmon originating from different maternal backgrounds. The following question was asked:

How does variation in egg size affect subsequent fitness in the wild?

It was expected that juveniles origination from larger, older females would have a higher survival and attain a larger body size due to maternal effects associated with egg size. Significant genotype- by- environment interactions on phenotypic traits were also expected, as bigger eggs would not always do better under all environments (Régnier *et al.*, 2013).

Chapter I

Maternal effects on embryo development in Atlantic salmon

Stringwell, R; Gutmann-Roberts, C; Price, E; Taylor, J; Gough, P. J. and Garcia de Leaniz, C. Maternal effects on embryo development in Atlantic salmon. (in prep.)

Chapter I

Maternal effects on embryo development in Atlantic salmon

ABSTRACT

Egg provisioning can greatly influence offspring fitness in many fishes, but the effects of maternal environment on offspring development are not clear. This study examined the variability in egg size, timing of hatching and emergence, and rate of yolk sac re-absorption of individually raised Atlantic salmon (*Salmo salar L.*) embryos from fertilization to yolk sac depletion. Rearing time of mothers in a hatchery environment significantly increased egg size after 26 months and also changed the relationship between timing of hatching and egg size. Small eggs from mothers which had only spent a short time in the hatchery environment hatched earlier than large eggs, a result commonly seen in wild salmonids. Yolk sac depletion was faster in embryos from mothers which had spent more time in the hatchery, suggesting an increased metabolic rate. Individuals with an increased metabolic rate are more likely to have an increased growth rate which can have direct consequences for key life history traits in the wild, such as timing of migration and maturation.

INTRODUCTION

Maternal traits are thought to be shaped by natural selection in response to environmental heterogeneity (Mousseau and Fox, 1998a; Einum and Fleming, 1999). Hence, the environmental variation experienced by mothers is translated into phenotypic variation in her offspring (Räsänen and Kruuk, 2007). When mothers have access to reliable cues about future environmental conditions, they may be able to predict the post-natal environment of their offspring and adjust their phenotype accordingly (Marshall and Uller, 2007; Segers and Taborsky, 2011). One of the most extensively studied aspects of maternal effects is egg size due to the positive correlation that often exists between embryo size, survival and fitness (Heath *et al.*, 1999; Einum and Fleming, 2000a).

Maternal fitness is a function of both fecundity and offspring fitness and so mothers are faced with a trade-off between offspring quantity and quality during reproduction (Berg *et al.*, 2001). Classic egg size theory predicts that organisms reproducing in a given environment should divide their available resources into eggs of an optimal size (Smith and Fretwell, 1974). This theory suggests that optimal egg size has evolved mainly in response to selection on maternal rather than offspring fitness (Einum and Fleming, 2000a). This concept does not, however, explain the large variation observed in egg size among females within a population, which is commonly correlated with female phenotype, for example larger, older and better condition females often produce larger eggs (Berg *et al.*, 2001; Christians, 2002; Einum and Fleming, 2002, Reid and Chaput, 2012). This suggests that optimal egg size is not necessarily a single, stable value but that there is adaptive phenotypic variation for egg size which may be explained by maternal effects (Einum and Fleming, 2002). Within female variation in egg size is also seen in a variety of organisms and this is often thought to be a method in which females ensure that at least a few offspring are of optimum size for a changing environment, commonly known as bet-hedging (Marshall *et al.*, 2008). That is, it may be more advantageous to female's fitness to have some success each year than having high success in some years and very low success in others. The bet-hedging theory predicts that unpredictable environments select for larger variance in within-clutch variation

(Gregersen *et al.*, 2009). Bet-hedging, however, is seldom selectively advantageous and when it is, then only for purely annual organisms (Einum and Fleming, 2004).

The relationship between metabolic rate and egg size suggests that development should be faster in smaller than in larger eggs (Valdimarsson *et al.*, 2002). As metabolic rate has a maternal influence and is directly related to maternal investment in egg mass, the egg size - survival relationship is considered a direct consequence of maternal effect on metabolic rate (Régnier *et al.*, 2010). This challenges the generally accepted view that 'bigger is better' and suggests that other maternal influences may promote within-population variability of egg size (Régnier *et al.*, 2013). The effects of egg size on an individual's fitness are likely to depend on the quality of the environment the individual faces, for example the benefits of large egg size are often observed in competitive environments, however in less competitive environments the advantages of producing large eggs are less evident (Einum and Fleming, 1999; Aprahamian *et al.*, 2003). Therefore, in highly competitve environments, both maternal and offspring fitness increase with per-offspring investment (Einum and Fleming, 1999), so that larger eggs may be favoured when juvenile density is high (Rollinson and Hutchings, 2010).

Several studies on fish have emphasised the benefits of developing from large eggs as they have more provisions and therefore more energy (Berg *et al.*, 2001). Individuals which hatch from small eggs can, however, compensate for the lack of maternal provisioning by either extending their developmental time or increasing their growth rate by increasing feeding rates and/or increasing conversion efficiency (Heath *et al.*, 1999). In some species of insects (e.g. the seed beetle (*Strator limbatus*)), individuals are able to extend development time in order to compensate for small size at hatching (Fox, 1997). This is not however an option for some species in which development time is constrained, for example in salmon, where the timing in which they must leave their natal stream is limited to a small window triggered by photoperiod and water temperature (Clarke and Shelbourn, 1985). The timing of such important niche shifts has been related to both developmental rates and energetic status (Forseth *et al.*, 1999). Thus, fish will move into a new environment if the growth and survival gains of utilizing a second habitat exceed the costs of moving between habitats (Gross, 1987). The provisioning provided by

mothers during early development and the offspring's individual energetic requirements may therefore condition offspring growth, with those from larger eggs having a competitive advantage over those from smaller eggs (Régnier *et al.*, 2012a).

For fish, increased maternal provisioning may not always be a benefit as larger eggs tend to take longer to hatch and therefore offspring from smaller eggs may be able to exploit food resources and access profitable feeding territories earlier (Garcia de Leaniz *et al.*, 2000; Valdimarsson *et al.*, 2002; Neely *et al.*, 2012). Such advantages may, however, be traded off against increased mortality due to reduced prey abundance and lack of predator dilution effects, (i.e. individuals reduce their per capita chance of attack, given that predator encounter probabilities do not increase in proportion to group size (Jaatinen and Öst, 2013)) which late emergers may experience (Einum and Fleming, 2000b). Alevins still dependent on their yolk sack represent energetically closed systems in which energy consumed from yolk is allocated among growth and metabolism (Kamler, 2008). Therefore, the rate at which the yolk-sac is absorbed and the allocation of yolk to development and metabolic energy are critical processes during early development (Barón-Aguilar *et al.*, 2013). Individuals with a higher metabolic rate demand have the potential to grow fast in favourable environments but at the cost of a high allocation of resources to routine metabolism, whereas those with a lower metabolic rate adopt a more conservative strategy with lower running costs but constrained growth (Millidine *et al.*, 2009). The trade-offs between growth and metabolism mean that variation in energy use will likely have implications for life-history traits and fitness (Burton *et al.*, 2011).

For salmonids such as the Atlantic salmon (*Salmo salar*), the early juvenile period is the most critical due to their complex life cycle. Like many fish species, offspring rely on maternal provisioning during these early stages making them suitable organisms to study variability in egg provisioning within and between clutches. Given that alevins must shift from endogenous to exogenous feeding, and that early growth and survival depend only on maternal provisioning and variation in individual assimilation, individual differences in energetic status and timing of niche shift are likely to be under selection (Andersson *et al.*, 2013). Salmonid species are also widely used in captive rearing programmes in order to supplement wild

populations and these practices are complicated by the phenotypic differences that may arise in captive compared to wild populations (Evans *et al.*, 2014a). As larger eggs produce larger offspring, it is often considered a benefit in hatcheries to have females which produce larger eggs but it is unclear how the effects of domestication influence early life development (Heath *et al.*, 2003; Neely *et al.*, 2012). This therefore leaves the question whether altering the rearing environment of mothers from the same genetic pool causes them to alter the investment they make into egg size and consequently the early life development of their offspring and whether there is an underlying effect of domestication in a hatchery environment which differs from that seen in the wild.

This study examined how variation in maternal provisioning affected embryo development. For this maternal provisioning was manipulated by rearing wild broodstock in a hatchery environment for increasing lengths of time to produce eggs of increasing sizes. Developmental traits of Atlantic salmon eggs (from eyed stage to hatch) and alevins (from hatch to exogenous feeding) within and between families were then analysed to provide insights into the evolution of maternal investment. The expectation was that wild females reared in the hatchery for longer would produce larger eggs, due to an increased supply of food, resulting in larger body size and therefore larger eggs. This would then give rise to offspring which hatch and emerge from the gravel later than those from smaller eggs. Utilization of yolk provisions was expected to be slower in offspring from females reared for longer in the hatchery due to an expected decrease in metabolic rate.

MATERIALS AND METHODS

Experimental fish

The study was conducted over two consecutive years, 2011-12 (Year 1) and 2012-13 (Year 2). Eggs were manually stripped from Atlantic salmon broodstock originating from the River Taff (South Wales) and held at the Natural Resources Wales, Cynrig Fish Culture Unit (Brecon, Wales). Female broodstock consisted of 12 (Year 1, n = 6; Year 2, n= 6) wild maiden fish (mean fork length 66.1 cm ± 6.1 SD), 12 (Year 1, n = 6; Year 2, n= 6) kelts (multiple spawners) reconditioned for 14 months in freshwater (mean fork length 68.8 cm ± 7.3 SD) and 10 (Year 1, n = 4; Year 2, n = 6) kelts reconditioned for 26 months in freshwater (mean fork length 75.5 cm ± 8.2 SD). Male broodstock consisted of 24 (Year 1, n = 12; Year 2, n = 12) wild males (mean fork length 66.2 cm ± 8.5 SD).

A split-brood breeding design was applied where two sires and three dams (one from each experimental group) were mated to produce 6 families in a 3 x 2 design (Fig. 1.1). The cross was replicated 6 times generating a total of 68 families (Year 1, n = 32, Year 2, n = 36). The eggs were incubated under standard hatchery conditions on a flow-through system at ambient temperature (Year 1, 6.7 °C ± 1.80 SD; Year 2, 5.9 °C ± 1.82 SD) and families were kept separated using modified incubation trays until eyed stage when they were then transported to a salmon recirculation system at Swansea University. Temperatures were recorded daily (0.1 °C) using a data logger and Degree Days (DDs) (a unit of measurement used to describe the number of thermal units accumulated over time where one DD equals 1 °C for 1 day) were used as a measure of development stage. One hundred eggs from each family were batch weighed (nearest 0.001g) to generate a mean and then individual eggs were categorized as either 'small' or 'large' for each given female depending on whether they were below or above the family mean.

Embryo development

Twenty-four individual eggs (12 "small" and 12 "large") from each family were weighed and assigned to individual 35 mm diameter wells within a six-well tissue culture plate (Nunc™) filled with 12ml of dechlorinated water. Each plate contained

3 "small" and 3 "large" eggs from the same female. Each egg was randomly allocated to a well within a plate and each plate was randomly allocated to a position on the bench by means of the "Randbetween" function in Excel in a constant temperature room at 9.4°C. Water was topped up every other day and changed weekly after hatching to prevent ammonia build-up. Mortalities and hatching events were recorded daily. Digital photographs (Canon EOS 400D, www.canon.com; 90 mm TAMRON SP Di 1:1 macro, www.tamron.eu/uk) of each individual were taken on the day of hatch and every week thereafter until full yolk-sac absorption. Photos were taken from a fixed distance against a standard background fitted with a scale bar. Yolk sac area was measured (to the nearest 0.005 mm) from the digital photographs using IMAGEJ (http://rsbweb.nih.gov/ij/).

Alevin emergence

Six emergence traps (artificial gravel nests) per female were set up following the design given in Daufresne *et al.*, (2005), three containing 20 'small' eggs and three containing 20 'large' eggs. The emergence traps were designed with a flow through nest compartment (6 cm, in diameter x 8.5 cm. long, mesh = 1 mm) where the eyed eggs were buried amongst gravel (size range: 10 - 28 mm) and a swim-up funnel for emerging alevins to pass through and become trapped in the collection chamber (6.5 cm in diameter x 9.5 cm long) (Fig. 1.2). The emergence traps were randomly placed in a flow-through hatchery trough (40 cm x 206 cm) which was fed via a recirculation system with a temperature logger installed in order to determine degree days. A light regime of 14 hours light 10 hours dark was employed during the experimental period. Emerging fish were recorded and removed daily at 10 AM and a digital photograph was taken to determine fry standard length and yolk sac volume at emergence using IMAGEJ. Traps were removed from the experiment if no fish had emerged after two months of the start date or if no other fish had emerged for 30 consecutive days from the last emergence.

Opercular beat rate

Opercular beat rate (OBR) was determined as a proxy for metabolic rate (Millidine *et al.*, 2008) for individual embryos during yolk absorption. OBR was determined by recording the time (seconds) taken for thirty opercular beats (repeated twice) and

then calculating beats min^{-1}. The degree day that the OBR was measured was also recorded. The fish contained in individual wells were observed in situ to minimise stress. OBRs were determined for individuals in Year 1 between 618 - 686 degree days and between a later developmental time of 914 - 970 degree days in Year 2 due to logistic constraints.

Statistical analysis

All analysis was carried out in R 3.0.0 using general linear mixed-effects models with the lme function from the 'nlme' package (Pinheiro *et al.*, 2007). Seven of the females used in the crosses for year 1 were used in the crosses for year 2 and so only the data collected for year 1 for those females were used in order to avoid unequal representation in the dataset. All tests were two-tailed with a significance level set to $\alpha = 0.05$. For model selection the step-down protocol was used where non-significant terms (interactions, main effects, and random effects) were backward eliminated, using Maximum Likelihood (ML) to check for significance. The final model was refitted using Restricted Maximum Likelihood (REML) following Crawley (2007). In the results section the t- and P-values are presented for all the fixed factors and interactions in the final model. Results from the initial models are presented in Appendix I (a-e). All post hoc tests between the female rearing groups were done using the ghlt function from the 'multcomp' package (Hothorn *et al.*, 2008) for multiple comparisons of the mean using Tukey contrasts. Data for egg weight, female fork length, yolk sac area and degree days were normalised with log_{10}-transformation.

Egg size

Egg weight was analysed with female rearing time (months), female body size (fork length) and their interaction as main effects. Female ID and year were included as random effects. Data collected in the second year for seven females that spawned on consecutive years were analysed as repeated measures to estimate individual female effects on egg size. For this, Female ID was included as a random effect and year was included as a main effect. The Fligner-Killeen test was used to assess homogeneity of variances in egg weight among females.

Timing of hatching

Timing of hatching (measured as degree days) was analysed with female rearing time (2, 14 and 26 months), egg weight (g) and egg size category (small/large) as main effects as well as the interaction between female rearing time and egg weight. Three females which had less than 60% hatch success were removed from the analysis. Female ID and year were included as random effects.

Yolk sac absorption

Analysis of yolk sac absorption (measured as the change in yolk sac area (mm) over time) included alevins that absorbed ≥75% of their yolk sac (n = 114) and excluded premature mortalities. Main effects included female rearing time (2, 14 and 16 months), egg weight (g), egg size category (small/large), developmental time (measured as degree days) and OBR. Interactions considered in the full model were female rearing time x developmental time, OBR x degree days the OBR was measured, female rearing time x egg weight, egg weight x OBR and female rearing time x OBR. Year, female ID and fry ID were included as random effects.

Timing of emergence

Timing of emergence (measured as degree days) was analysed with female rearing time (2, 14 and 26 months), egg weight (g) and egg size category (small/large) as main effects. The interaction between female rearing time x egg weight was also considered. Year, Female ID and trap ID were included as random effects.

Yolk sac reserves at emergence

Yolk sac reserves at emergence (measured as yolk sac area (mm)) were analysed with female rearing time (2, 14 and 26 months), egg weight (g), egg size category (small/large) as main effects. Degree day of emergence was also included as a covariate in the model. Interactions considered in the full model were female rearing time x egg weight, female rearing time x timing of emergence and egg weight x timing of emergence Year, Female ID and trap ID were included as random effects.

RESULTS

Egg size

An overall positive effect of time females spent in the hatchery on egg weight was found ($F_{2,21}$ = 8.43, P = 0.002). Egg weight was significantly larger for females which spent 26 months in the hatchery than those which were only retained for 2 - 14 months (Table 1.1a, Fig. 1.3a). An overall positive effect of female body size on the size of eggs was detected (Table 1.1a) and female ID accounted for 81.93% of the variation in egg size. A significant interaction between female rearing time and body size on egg weight was detected, whereby the relationship between female body size and egg size differed for females kept in the hatchery for 14 months compared to those that had been there for only 2 months (Table 1.1a, Fig. 1.3b). No difference was detected for females kept in the hatchery for 26 months (Table 1.1a). Pairwise comparisons indicated a significant difference between 2 months and 26 months (z = 2.51, P = 0.032) and between 14 months and 26 months (z = 2.36, P = 0.047) but no significant difference between 2 months and 14 months (z = 0.18, P = 0.981).

For the seven females examined in both experimental years, egg weight significantly increased with female fork length (Table 1.1b, Fig. 1.4). There was also a significant effect of the year the experiment was carried out on egg size and a significant interaction between year and female fork length with the effect of female body size being greater in year 2 to that in year 1 (Table 1.1b).

Results from the Fligner-Killeen tests revealed a significant difference in egg size variation among the three groups of females (χ^2_2 = 17.93, P = 0.001). Females reared in the hatchery for only two months had a coefficient of variation (CV %) of 16.1%. Females reared in the hatchery for 14 months had the highest CV of 21.3% and females reared in the hatchery for 26 months had a CV of only 11.6%.

Timing of hatching

Egg weight had a significant negative effect on timing of hatch (Table 1.2). No effect of female rearing time was detected ($F_{2,20}$ = 2.72, P = 0.090, Appendix I- a), however a significant interaction between female rearing time and egg weight on timing of hatching was found (Table 1.2). Offspring from mothers which had been in the

hatchery for only two months were hatching earlier when eggs were smaller, offspring from mothers which had been retained in the hatchery for 14 months were hatching at the same time and those from mothers which had spent 26 months in the hatchery were hatching earlier when eggs were large (Fig. 1.5). No effect of egg size category was detected so was removed from the model (Appendix I - a). Year accounted for 96.7% of the variability in the timing of hatching while female identity accounted for only 1.08%. Pairwise comparisons indicated no significant difference between 2 months and 14 months ($z = -0.12$, $P = 0.992$), 2 months and 26 months ($z = -1.80$, $P = 0.167$) and 14 months and 26 months ($z = -1.68$, $P = 0.209$).

Yolk sac reabsorption

A positive effect of egg weight on yolk sac reserves was found along with a negative effect of developmental time (degree days) (Table 1.3). Female rearing time had an overall significant effect on yolk sac size ($F_{2,11} = 53.09$, $P < 0.001$). Yolk sac size was significantly larger for offspring from females which spent 26 months in the hatchery than those which were only retained for 2 months (Table 1.3; Fig. 1.6). A significant interaction between female rearing time and degree days was also detected, whereby offspring from females which had spent longer periods of time in the hatchery had larger yolk sacs at hatching but utilized the resources quicker than alevins from mothers which had only spent 2 months in the hatchery (Table 1.3, Fig. 1.6). Offspring from mothers which spent 26 months and 14 months in the hatchery fully absorbed their yolk reserves (YS = 0) at approximately 1140 and 1057 degree days respectively, whereas those from mothers which had only spent 2 months in the hatchery took approximately 1223 degree days. Opercular beat rate and egg size category had no effect and so were removed from the model along with non-significant interactions (Appendix I-b). Year accounted for 20.5% of the variation in yolk sac and female identity accounted for 2.9% of the variation. Pairwise comparisons indicated significant differences in yolk sac size between 2 months and 26 months ($z = 3.24$, $P = 0.003$) and 14 months and 26 months ($z = 2.52$, $P = 0.031$) but no significant difference between 2 months and 14 months ($z = 1.09$, $P = 0.519$).

Timing of emergence

The timing of emergence from the nest was not significantly explained by any of the main effects (Appendix I-c). Year accounted for 17.1% of the variability, female identity accounted for 9.8% of the variability and trap accounted for 36.7 % of the variability.

When fry emerged from the nest, yolk sac reserves were positively related to egg size and negatively related to timing of emergence (Table 1.4), so that those alevins which emerged earlier from the nest had larger yolk sacs. No effect of female rearing time or egg size category was detected and so both were removed from the model along with non-significant interactions (Appendix I-d). Year accounted for 30.7% of the variation in yolk sac reserves, female identity accounted for 19.9% of the variability and trap identity accounted for 4.2% of the variability.

DISCUSSION

Temperature, parental effects and egg size or egg quality are all known to influence offspring development rates in fish (Geffen and Nash, 2012). There have been a number of studies examining the relationship between egg size and the rate of development in fish, generally focussing on the time elapsing between fertilization and hatching as a measure of developmental rate (Valdimarsson *et al.*, 2002). A study examining 140 species of marine fish concluded that time from fertilization to hatching was positively related to egg size (Pauly and Pullin, 1988). Other studies looking at the effect of egg size on development within a species have not found any relationship between egg size and time to hatch (Pepin *et al.*, 1997; Einum and Fleming, 1999). Given the negative relationship between metabolic rate and size (larger eggs or embryos have proportionately lower metabolic rates), it is considered that smaller eggs should develop faster than larger eggs and so this apparent lack of consistent relationship between egg size and developmental rate in fishes is surprising (Sargent *et al.*, 1987). The present study looked at the development rate of individual embryos from fertilization to emergence from the nest and demonstrates that variation in rearing environment experienced by the mothers can alter the maternal investment into egg size and the developmental rate of the offspring.

Mothers that spent 26 months in the hatchery produced larger eggs than did maiden hens which had only been in the hatchery for two months when statistically controlling for female body size. An overall positive relationship between female body size and egg weight was found in this study. However, although egg weight increased with female body size in maiden fish, which is typically found in wild Atlantic salmon (Hendry *et al.*, 2001; Rollinson and Hutchings, 2011), this relationship was weakened for kelts. In hatchery conditions, natural selection which typically favours large eggs in the wild, is relaxed allowing fecundity selection to drive the rapid evolution of small eggs (Heath *et al.*, 2003). In our study however, females were producing larger eggs for their given body size as they spent longer in the hatchery. The observations seen here contradict the model proposed by Hendry *et al.*, (2001) for sockeye salmon (*Oncorhynchus nerka*) which predicted that in profitable environments an increase in female size should result in greater proportional increases in egg number rather than egg size.

Variation in egg size within females in each rearing group was found, however there is some evidence for the strategy of diversified bet-hedging in females which have been reared in captivity for 14 months as an increase in variation was observed in comparison with maiden females. Variation then almost halved in females which had spent 26 months in captivity, which was less variable than maiden females - a possible response to a consistent environment. This could be suggestive of conservative bet-hedging, where fewer and larger offspring are produced (Einum and Fleming, 1999). However, as variation between both groups of kelts differed it is likely that the increased variation in egg size observed in females held in the hatchery for 14 months was a physiological constraint in the ability to produce equally sized eggs, which may be influenced by the females' physiological status (Einum and Fleming, 2004).

Plasticity in timing of hatching has been documented in a number of animals in response to predators, pathogens, conspecifics and food availability (Warkentin, 2011). As with many other important life history switch points, plasticity in timing of hatching may have carryover effects (Warkentin, 2011). According to life-history theory, increased risk of larval mortality should favour delayed hatching, while relatively high egg mortality should favour early hatching (Wedekind and Müller, 2005). The relationship between egg size and timing of hatching in salmonids has been well studied and many have found that there is no correlation (Beacham *et al.*, 1985; Hutchings, 1991; Einum and Fleming, 1999). In the present study it was found that egg size was correlated with the timing of hatching, with large eggs hatching earlier than small eggs overall. In the wild, there is a trade-off for the timing of hatching, as those which hatch early and consume provisions quickly may face an increased risk of predation due to negative frequency dependant effects, whereas late hatching and slower consumption of resources may reduce the chance of acquiring a profitable feeding territory (Brännäs 1995; Einum and Fleming, 2000b). Small fry often compensate for the risks associated with early hatching and emergence with an increased growth rate, metabolic rate and aggressive behaviour (Heath *et al.*, 1999). In this study, it was observed that small eggs from maiden females hatched earlier than large eggs, which supports predictions from life-history theory, in relation to perceived risk of larvae predation (Capellán and Nicieza, 2007). However, in kelts

there was a tendency for large eggs to hatch at the same time or earlier than small eggs.

As there is no predation in the hatchery environment, selection for fast growth is likely to occur (Heath *et al.*, 1999) and so it may be beneficial for large eggs to hatch earlier than small eggs. Whether the differences in hatching timing have fitness implications is uncertain. Timing differences could also be related to juvenile densities as kelts had higher egg densities which would create greater competition for space and food at emergence (Elliott, 1986). This could lead to selection for earlier hatching of large eggs if fry emerging first have a competitive advantage in relation to social dominance and growth rate (Donaghy and Verspoor, 1997).

In this study, offspring from kelts which had spent 26 months in the hatchery hatched with more maternal provisions than those from maiden females and females which had been in the hatchery for 14 months and this was also positively associated with egg weight. It has been documented that small embryos complete development faster than large embryos (Gillooly *et al.*, 2002). Interestingly, however, offspring from kelts and larger eggs absorbed their yolk provisions at a faster rate, exhausting their yolk sac reserves up to 166 degrees days earlier than maiden female offspring. In contrast, those offspring from wild mothers absorbed their yolk sac at a much slower rate which would provide longer protection in the spawning nest. Individuals with a higher energetic content tend to deplete their resources faster due to a higher metabolic rate (Régnier *et al.*, 2012b). No association between ventilation frequency and yolk reserves were, however, found in this study which may be due to the inherent difficulty of observing opercula movement on newly hatched alevins in a confined environment. Metabolic rate is under maternal influence and appears to be related to maternal investment in salmonids, with larger fry typically having lower metabolic rates (Régnier *et al.*, 2013). The consequences for variation in metabolic rate can be substantial and have been linked with traits such as growth and survival (Burton *et al.*, 2011). Individuals with a higher metabolic rate incur greater energy costs than those with a lower metabolic rate and may therefore have a greater potential for food processing and growth when food is abundant (Millidine *et al.*, 2009).

Timing of emergence appears to be under selection in salmonids (Einum and Fleming, 2000a). It is thought that small offspring which typically develop quicker may gain an advantage over large offspring due to earlier access to feeding territories and a prior residence effect (Johnsson *et al.*, 1999; Rollinson and Hutchings, 2010). Some studies have found that timing of emergence is positively related to egg size as offspring from larger eggs emerge later than those from smaller eggs (Rollinson and Hutchings, 2011). The present study, however, found no effect of egg size or maternal rearing environment on the timing of emergence, a result also observed by Einum and Fleming (1999). Instead, a large effect of emergence trap identity (37%) was found to explain the variation in emergence time from the nest. It is predicted that emergence from the gravel should be well synchronised in salmonid fry due to dilution effects against predation (Brännäs, 1995). The large effect of trap identity on the timing of emergence seen in this study, whereby regardless of egg size and other predicting factors fish emerged from the nest in synchronisation, validates this theory. However, what drives the first individual to emerge from the nest may be influenced by factors which in this study have been confounded by this synchronous effect. Juvenile Atlantic salmon exhibit a normally distributed temporal pattern of emergence with a duration of approximately two weeks, although the majority tend to emerge synchronously over a three-night period (Cutts *et al.*, 1999). Early emerging juveniles have a competitive advantage over later emerging conspecifics by being first to acquire the available territorial space (Metcalfe and Thorpe, 1992). A confounding variable in explaining any links between maternal effects and emergence timing may therefore be competition for prior residence of a territory. When the amount of yolk remaining at the time of emergence was considered there was a negative relationship between emergence date and individual yolk sac size. This relationship is a likely consequence of time spent in the gravel, with early emerging fry having more un-metabolised yolk than later emerging conspecifics (Garcia de Leaniz *et al.*, 2000; Régnier *et al.*, 2012b). This therefore suggests that individuals do not emerge because of an energy shortage imposed by their metabolic requirements (Régnier *et al.*, 2012b).

The timing of niche shifts can be critical to survival, and plasticity at these events can clearly be advantageous (Warkentin, 1995). Rearing females in a hatchery environment for increasing amounts of time significantly increased egg investment

and shifted the relationship between egg size and timing of hatch. Additionally a change in metabolic rate likely occurred in this study, as offspring originating from females retained in the hatchery consumed their yolk sacs at a much faster rate. Ultimately, an increased metabolic rate can have an effect on later life history thresholds in salmonids, such as timing of migration and maturation (Wilke *et al.*, 2014). By using individual level measurements of timing of hatch and yolk sac utilization, this study has shown that the rearing environment of mothers can alter the timing of important life stages.

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Table 1.1. Results of linear mixed-effects models examining a) the influence of body size and time Atlantic salmon females (n = 27) spent in hatchery environment on egg weight (n = 1296). Pairwise comparisons indicated a significant difference between 2 months and 26 months (z = 2.51, P = 0.032) and between 14 months and 26 months (z = 2.36, P = 0.047) but no significant difference between 2 months and 14 months (z = 0.18, P = 0.981); and b) the influence of body size on egg weight analysed as repeated measures of Atlantic salmon females (n = 7) examined in two consecutive years. Estimates of the magnitude of the effect of each parameter on egg weight (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
a) All females					
Intercept	-0.945	0.021	1269	-45.448	**< 0.001**
14 months	0.005	0.030	21	0.183	0.856
26 months	0.099	0.040	21	2.510	**0.020**
Female FL	0.060	0.024	21	2.522	**0.020**
14 months x Female FL	-0.097	0.032	21	-2.985	**0.007**
26 months x Female FL	-0.054	0.036	21	-1.502	0.148
b) Seven repeated females					
Intercept	-8.519	0.755	607	-11.277	**< 0.001**
Female FL	2.663	0.266	607	10.012	**< 0.001**
Year 2	-4.246	0.240	607	-17.670	**< 0.001**
Female FL x Year 2	1.500	0.0840	607	17.865	**< 0.001**

Table 1.2. Results of linear mixed-effects models examining the influences on the timing of hatching of Atlantic salmon alevins (n = 1096) originating from females which had spent varying lengths of time in the hatchery (n = 24). Estimates of the magnitude of the effect of each parameter on timing of hatching (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced. Pairwise comparisons indicated no significant difference between 2 months and 14 months (z = -0.12, P = 0.992), 2 months and 26 months (z = -1.80, P = 0.167) and 14 months and 26 months (z = -1.68, P = 0.209).

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	2.705	0.043	1069	63.130	**< 0.001**
14 months	-0.0003	0.003	20	-0.120	0.906
26 months	-0.007	0.004	20	-1.803	0.087
Egg Weight	-0.005	0.001	1069	-4.169	**< 0.001**
14 months x Egg Weight	0.003	0.002	1069	20.147	**0.032**
26 months x Egg Weight	0.006	0.002	1069	3.019	**0.003**

Table 1.3. Results of linear mixed-effects models examining the influences on yolk sac absorption of Atlantic salmon alevins (n = 114) originating from females which had spent varying lengths of time in the hatchery (n = 15). Estimates of the magnitude of the effect of each parameter on yolk sac area (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced. Pairwise comparisons indicated significant differences between 2 months and 26 months (z = 3.24, P = 0.003) and 14 months and 26 months (z = 2.52, P = 0.031) but no significant difference between 2 months and 14 months (z = 1.09, P = 0.519).

Parameter	**B**	**βSE**	**DF**	**t**	**p**
Intercept	24.480	2.027	516	12.077	**< 0.001**
14 months	0.988	0.909	11	1.088	0.300
26 months	3.847	1.186	11	3.243	**0.008**
Developmental time	-11.085	0.399	516	-27.776	**< 0.001**
Egg Weight	2.718	0.425	98	6.401	**< 0.001**
14 months x Developmental time	-2.177	0.507	516	-4.289	**< 0.001**
26 months x Developmental time	-4.599	0.596	516	-7.717	**< 0.001**

Table 1.4. Results of linear mixed-effects models examining the influences on yolk sac reserves at the time of emergence from the gravel of juvenile Atlantic salmon (n = 1988) originating from females which had spent varying lengths of time in the hatchery (n = 25). Estimates of the magnitude of the effect of each parameter on yolk sac area (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	0.866	0.081	1843	10.632	**< 0.001**
Egg Weight	0.115	0.016	118	7.112	**< 0.001**
Timing of emergence	-0.150	0.004	1843	-37.463	**< 0.001**

Figure 1.1.

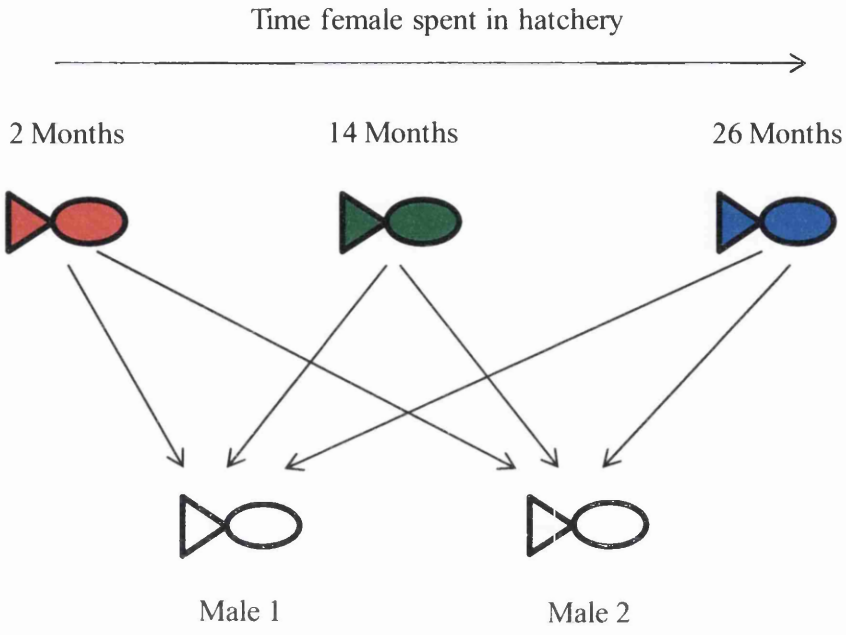

Figure 1.1. Schematic diagram of 3 x 2 split-brood breeding design. Two male Atlantic salmon were crossed with three females (one from each experimental group; 2 months in hatchery (●), 14 months in hatchery (●) and 26 months in hatchery (●) to produce 6 families.

Figure 1.2.

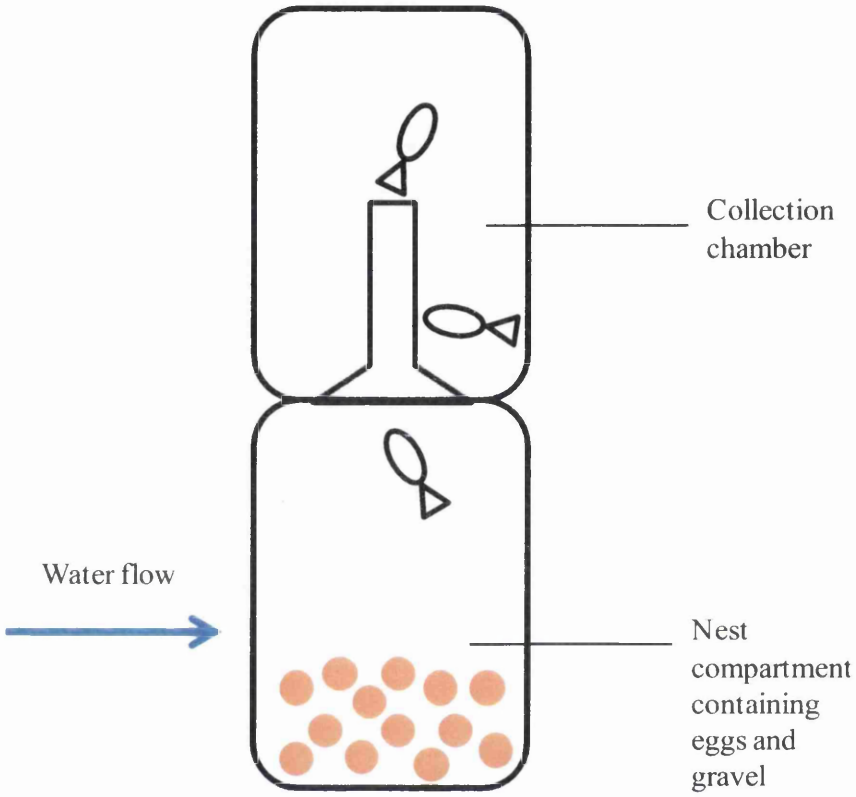

Figure 1.2. Schematic diagram of the Atlantic salmon emergence traps (used to check the timing of emergence of the fish). The traps were made of two connected cylinders; the nest compartment (6 cm diameter x 9.5 cm long, mesh netting 1 mm) filled with gravel where twenty eggs were buried; and an upper removable collection chamber (6.5 cm diameter x 9.5 cm long) in which fish were trapped as they emerged from the gravel.

Figure 1.3.

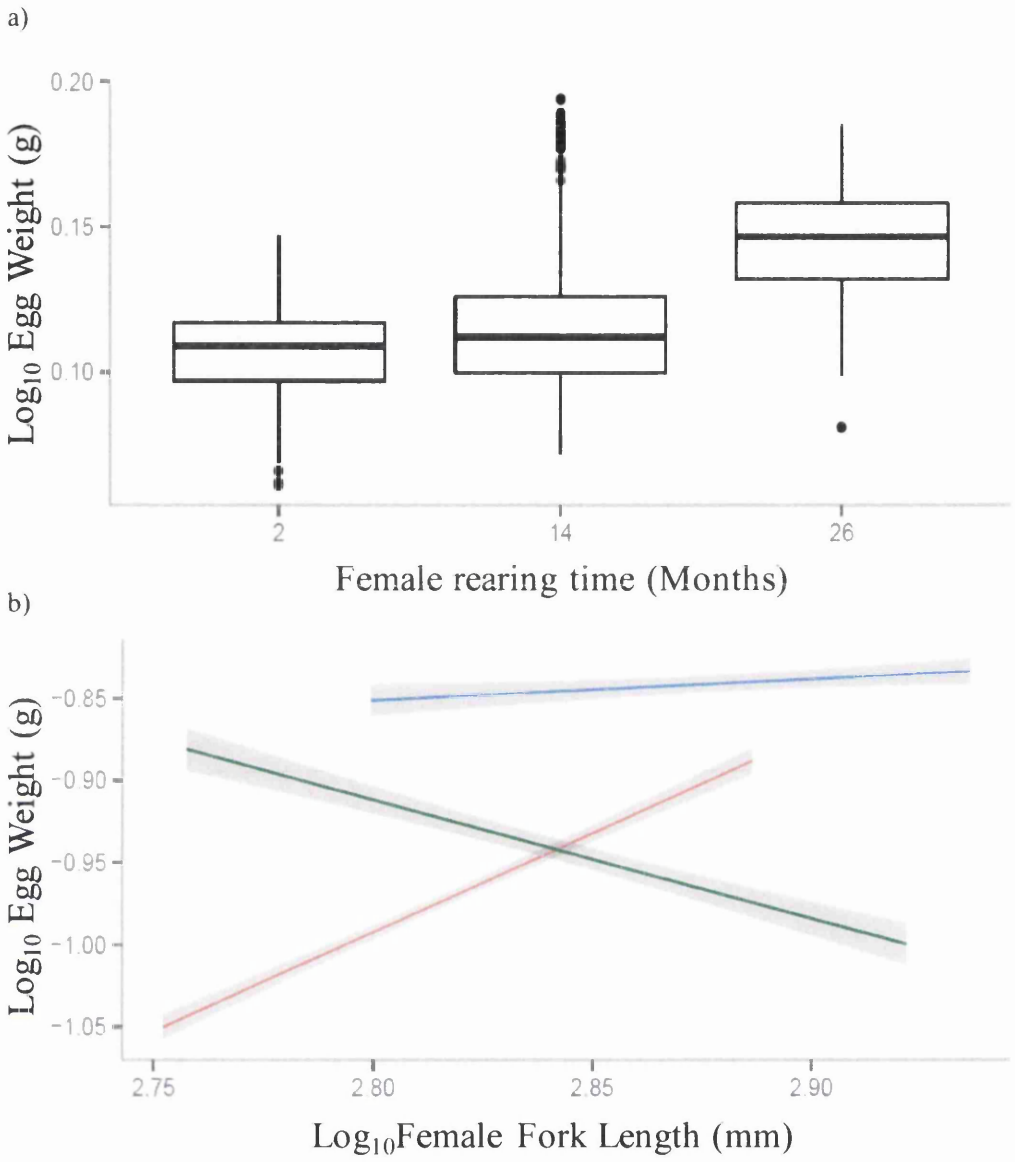

Figure 1.3. a) Variation in female Atlantic salmon egg weights (g) for each experimental rearing group; 2 months in hatchery (n = 12), 14 months in hatchery (n = 9) and 26 months in hatchery (n = 6). The boxplots show medians, upper and lower quartiles, and range (outliers indicated by dots) indicated by line, box, and error bars, respectively. b) Effect of female body size (log_{10} fork length, mm) on egg size (log_{10} egg weight, g) in wild female Atlantic salmon reared in a hatchery environment for 2 months (Red), 14 months (Green) and 26 months (Blue). Line represents linear mixed-effects model fit of the data. Grey shading represents 95% confidence intervals. Results from linear mixed effects model are given in Table 1.1a.

Figure 1.4.

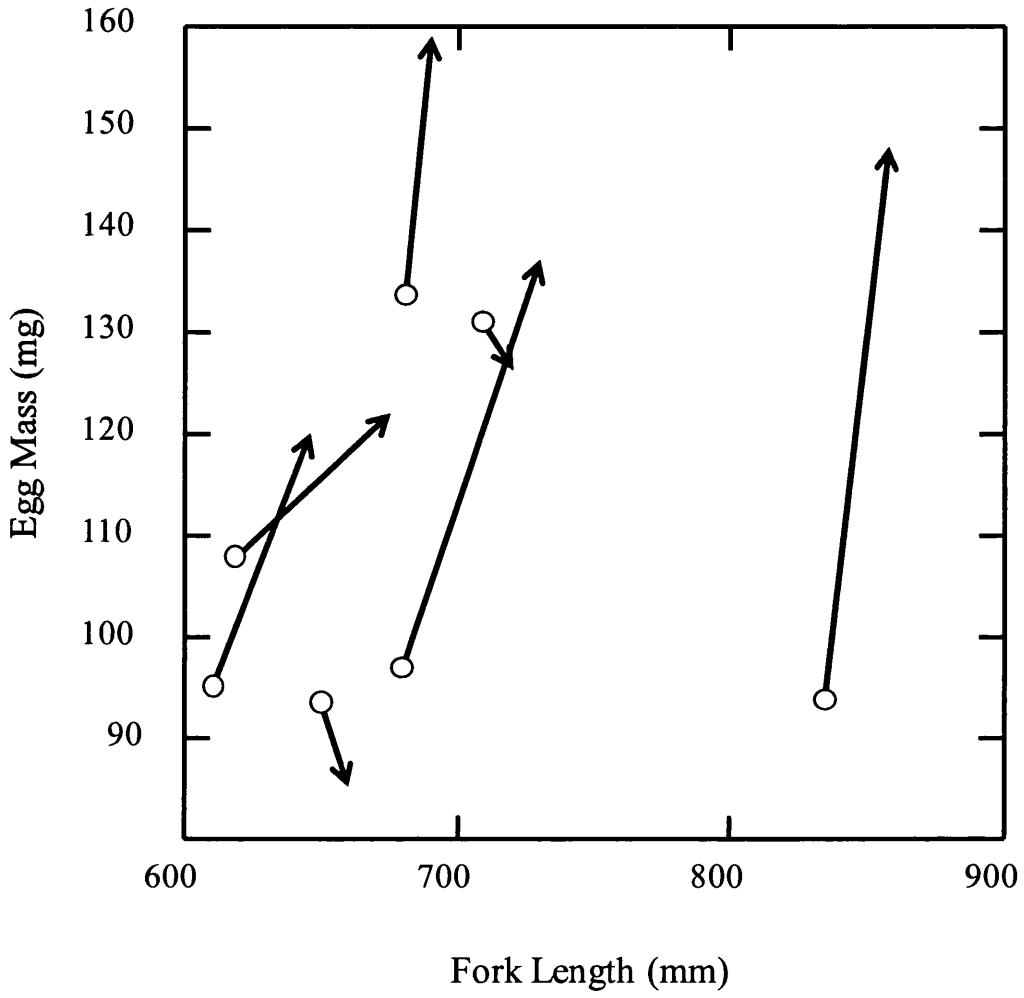

Figure 1.4. Relationship between female Atlantic salmon body size (fork length, mm) and mean egg size (weight, mg) for seven females which were examined for two consecutive years. Arrows depict direction of change of egg weight from the first year to the second year of experiment for each individual female. Results from linear mixed effects model of repeated measures are given in Table 1.1b.

Figure 1.5.

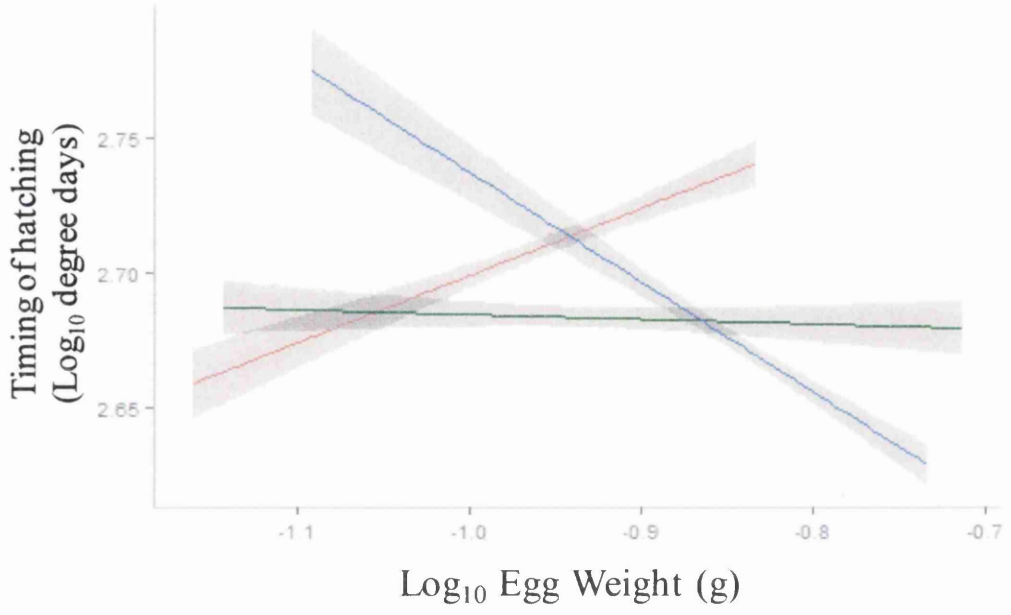

Figure 1.5. Effect of egg size (log_{10} egg weight, g) on timing of hatching (log_{10} degree days) in offspring from wild female Atlantic salmon reared in a hatchery environment for 2 months (Red), 14 months (Green) and 26 months (Blue). Line represents linear mixed-effects model fit of the data. Grey shading represents 95% confidence intervals. Results from linear mixed effects model are given in Table 1.2.

Figure 1.6.

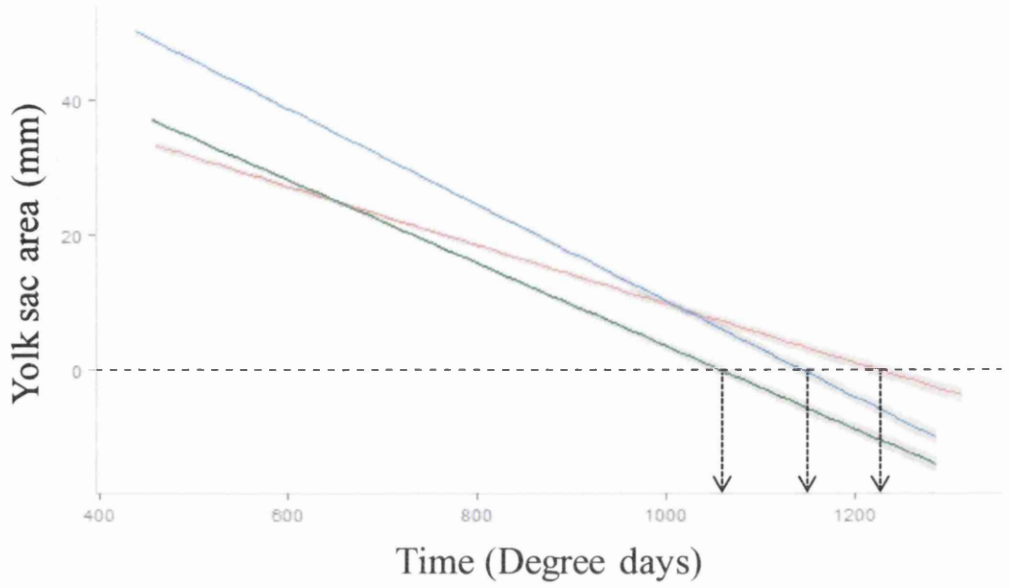

Figure 1.6. Effect of development time (degree days) on yolk sac absorption (yolk sac area, mm) in offspring from wild female Atlantic salmon reared in a hatchery environment for 2 months (Red), 14 months (Green) and 26 months (Blue). Line represents linear mixed-effects model fit of the data. Grey shading represents 95% confidence intervals. Results from linear mixed effects model are given in Table 1.4.

Chapter II

Maternal effects on early behaviour in Atlantic salmon

Stringwell, R; Taylor, J; Gough, P. J. and Garcia de Leaniz, C. Maternal effects on early behaviour in Atlantic salmon. (in prep.)

Chapter II

Maternal effects on early behaviour in Atlantic salmon

ABSTRACT

Correlations between behavioural and physiological traits, often termed stress coping styles, are widespread in a number of animal groups. Such trait variation can generally be classified into two distinct groups with animals characterized as either proactive or reactive. Salmonid alevins are dependent on the quality of maternal investment for growth and metabolism, which can also influence the timing of emergence. Emergence from the spawning redd after the depletion of maternally derived nutrients is a crucial niche shift for salmonid fry and the timing of this event may be expected to be correlated to behavioural and physiological traits such as aggression and metabolic rate. The present study examined the relation between maternal provisioning, timing of emergence, aggression and metabolic rate in newly emerged Atlantic salmon fry. Maternal provisioning was manipulated by varying the length of time wild mothers spent in captivity. Early emerging individuals showed a higher level of aggression in comparison with those which emerged later, consistent with a proactive coping style. However, ventilation frequency (a proxy for metabolic rate) was not correlated with the timing of emergence or aggression, suggesting there is an uncoupling of stress coping styles in the hatchery environment. Aggression was inversely related to the time mothers spent in the hatchery, suggesting that when maternal provisioning is high, aggression may be selected against.

INTRODUCTION

Mothers may influence the timing of ontogenetic larval niche shifts by affecting the energy stores (maternal investment) and metabolic rate of their offspring (Régnier *et al.*, 2012b). In salmonids, the time at which fry emerge from the safety of the gravel bed in search of exogenous feed and suitable territories is a critical life history event (Einum and Fleming, 2000a; Andersson and Höglund, 2012), characterized by high mortality rates (Coughlin, 1991). The timing of emergence and the ability to establish a new territory are therefore traits likely to be under selection, as competitive ability of fry in the first few months post-emergence is essential to their survival (Einum and Fleming, 2000b; Åberg Andersson *et al.*, 2011). Dominant fish typically obtain the most energetically profitable feeding territories (Berejikian, 1995; Berejikian *et al.*, 1996) and individuals that fail to acquire a suitable territory are more likely to starve or show reduced growth rates (Cutts *et al.*, 1999).

Variation in body size at first feeding amongst fry is an important determinant of success as many studies have proposed that larger individuals have a competitive advantage in dominance encounters, and obtain food faster than smaller individuals (Berejikian, 1995; Benhaim, 2003). However, it seems that the earliest salmonid fry to emerge from the redd are able to gain a prior residence advantage of feeding territories and therefore may be more dominant and successful in competitive interactions in order to defend their territory (Andersson *et al.*, 2013). This prior residence then enables the fish to establish and maintain a size advantage (Metcalfe and Thorpe, 1992; Huntingford and Garcia de Leaniz, 1997; Andersson *et al.*, 2013). For example, Skoglund *et al.* (2011) manipulated the development timing of Atlantic salmon (*Salmo salar*) fry and found that early-emerging individuals released into the wild had both higher survival and larger final body size than individuals emerging later.

Maternal effects, whereby the female's phenotype or environment influence the phenotype of the offspring, can have underlying consequences on juvenile behaviours and life-history traits which are important to fitness (Einum and Fleming, 2000b). For example, when female three-spined stickleback (*Gasterosteus aculeatus*) were exposed to the threat of predation they produced offspring which exhibited tighter shoaling behaviour (an anti-predator response), than offspring from non-

exposed females (Giesing *et al.*, 2011). A direct effect of the rearing environment experienced by the mother is the size of eggs she is able to produce as the amount of resource she can allocate to her progeny may be limited if food resources are low (Bernado, 1996). For example, the nutritional status of the female can influence gonadal development and limit the amount and the quality of the eggs she produces (Johnston *et al.*, 2007). Conversely, female African cichlids (*Simochromis pleurospilus)* raised in a poor environment as juveniles produced larger young than females raised without food limitations, suggesting that mothers have the ability to prepare their offspring for similar environmental conditions (Taborsky, 2006). Egg size is a widely studied maternal investment due to its direct consequences for offspring growth, fitness and survival (Einum and Fleming, 1999; Höjesjö *et al.*, 2011).

Metabolic rate has been directly correlated to behaviour as individuals with a high metabolic rate have higher dominance ranks and exhibit more aggressive behaviour than those with a lower metabolic rate (Metcalfe and Thorpe, 1992). This is considered to be a result of higher growth and metabolic rates requiring greater energetic demands and so individuals tend to exhibit more aggression and engage in more exploratory behaviour which facilitate resource acquisition (Edenbrow and Croft, 2013). An increased metabolic rate has also been associated to the transfer of hormones from mother to embryo (Burton *et al.*, 2011). For example, brown trout (*Salmo trutta*) embryos exposed to maternally derived stress cortisol displayed elevated oxygen consumption rates during development and also exhibited more aggressive behaviour than those which were not exposed (Sloman, 2010).

The individual differences in the way animals respond to stressful situations are often referred to as behavioural syndromes, coping styles, temperaments or animal personalities (Wolf *et al.*, 2007). The term stress coping styles is often used to describe suits of behavioural and physiological responses to challenges that are constant over time (Koolhaas *et al.*, 1999). Such behavioural and physiological differences have been clustered into two characteristic responses, termed proactive and reactive stress coping styles (Koolhaas *et al.*, 1999; Vaz-Serrano *et al.*, 2011). Early-emerging salmonid fry tend to exhibit a more proactive stress coping style and late-emergers a more reactive coping style (Andersson *et al.*, 2013). Proactive individuals typically display more aggressive behaviour with an increased "fight or

flight" stress response and a lower parasympathetic reactivity (ability to return to homeostasis after experiencing stress) than their reactive counterparts (Verbeek *et al.*, 2008; Martins *et al.*, 2011). This increased response to stress (manifested by an increase in ventilatory frequency) may be an adaptive response that enables the individual to prepare for possible physical responses to a competitor or predator (Hawkins *et al.*, 2004; Roberts and Garcia de Leaniz, 2011).

An uncoupling between correlated behavioural and physiological characteristics of stress coping styles has been observed in hatchery environments, thought to be a consequence of domestication (Johnsson *et al.*, 2001; Vaz-Serrano *et al.*, 2011; Huntingford *et al.*, 2012). In the wild, a lack of feeding territories, presence of predators, competition from conspecifics and variable environmental conditions could result in the co-selection of the suits of traits that form the stress coping styles (Øverli *et al.*, 2007). However, in hatchery-reared fish this directional selection during emergence is absent, and so variation in behaviour is likely to increase (Endler, 1986). A number of studies comparing hatchery fish with wild fish have reported that hatchery reared salmonids and their offspring were more aggressive than their wild counterparts (Einum and Fleming, 1997; Berejikian *et al.*, 1999). Several hypothesis have been proposed to explain why this might be, including: the high densities of fish in hatcheries can suppress the establishment of social dominance that would occur in natural streams, and therefore promote high aggression in hatchery fish once released (Weber and Fausch, 2003); and physiological characteristics of hatchery fish might also influence aggression as selection for faster-growing fish by hatcheries correlates with higher levels of growth hormone, which can increase aggressive behaviour (Johnsson and Björnsson, 1994). Additionally, maternal effects could also play a crucial role at this early stage of development as alevins have been dependant on the maternally derived nutrients which may have been affected by the physiological status of the female. How maternal environment affects the relationship between early behaviour and physiology has yet to be determined.

A split-brood breeding design was employed in order to investigate behavioural and physiological patterns in newly emerged juvenile Atlantic salmon from different maternal backgrounds. For this, maternal provisioning was manipulated by varying the length of time mothers from the same genetic

background were fed in captivity, two months, 14 months and 26 months, which resulted in three groups of eggs of increasing size. In order to control for the effect of environment and social experience, behaviour was compared among juveniles reared in the same environment from fertilization and each isolated from other individuals at emergence. The expectation was that aggression would increase with a) longer time spent in the hatchery by the mother, due to an inadvertent selection for domesticated traits, b) body size and c) early timing of emergence and increased metabolic rate, corresponding with the proactive stress coping style.

METHODS AND MATERIALS

Experimental fish

Eggs were manually stripped from Atlantic salmon broodstock originating from the River Taff (South Wales) and held at the Natural Resources Wales, Cynrig Fish Culture Unit (Brecon, Wales) in December 2012. Female broodstock consisted of six wild maidens (mean fork length 69.8 cm ± 5.6 SD), six kelts reconditioned for one year in freshwater (mean fork length 68.1 cm ± 6.7 SD) and six kelts reconditioned for two years in freshwater (mean fork length 75.9 cm ± 8.1 SD). Male broodstock consisted of twelve wild males (mean fork length 68.3 cm ± 11.0 SD). A split - brood breeding design was applied where two sires and three dams (one from each experimental group) were mated to produce 6 families in a 3 x 2 design (Fig. 1.1). The cross was replicated 6 times generating a total of 36 families.

Eggs were incubated under standard hatchery conditions on a flow-through system at ambient temperature (5.8 °C ± 1.91 SD) until eyed stage when they were then transported to the recirculation system at Swansea University. Temperatures were recorded daily using a data logger and cumulative thermal units were used as a measure of development stage. Eggs from each female (and two respective males) were batch weighed (nearest 0.001g) to generate a mean and then individual eggs were categorized as either 'small' or 'large' for each given female depending on whether they were below or above the family mean. Six emergence traps (Fig. 1.2) adapted from Daufresne *et al.*, 2005 [Chapter 1] per female were set up and consisted of batches of 20 eggs - three with 'small' eggs and three with 'large' eggs. Mean egg weight for each nest was also recorded. On the day of emergence (recorded as degree day of emergence) a digital photograph was taken to determine fry standard length (nearest 0.005 mm) using ImageJ before the fish was housed in an individual flow-through container (4cm x 4cm). Behavioural observations took place between 1 - 5 days after an individual emerged from the gravel nest. A total of 299 individuals were randomly selected for behavioural testing between 18th March and 26th April 2013.

Metabolic rate

Opercular beat rate (OBR) was recorded before the fish were examined for behavioural traits as a proxy for metabolic rate (Millidine *et al.*, 2008). OBR was determined by recording the time (seconds) taken for thirty opercula beats (repeated twice) and then calculating beats min^{-1}. This was carried out before the fish was removed from the flow-through container to avoid exerting additional stress on the individual.

Mirror image stimulation

Mirror image stimulation (MIS) experiments (Gallup Jr, 1968; Johnsson *et al.*, 2003; Höjesjö *et al.*, 2011) were conducted in order to quantify agonistic behaviour without the requirements needed for dyadic encounters such as size matching and eliminating bias from responses to kin and nonkin (Brown and Brown, 1993; Berejikian, 1995). MIS tests have been criticized for being unrepresentative of agonistic relationships in the wild as fish always encounter an equally sized opponent which provides unusual visual feedback (Ruzzante, 1992; Rowland, 1999). However, in newly emerged salmonids, reactions to mirror images have been shown to correlate positively with social dominance in paired contests with conspecifics (Holtby *et al.*, 1993 Berejikian *et al.*, 1996). The testing system (Fig. 2.1) consisted of twelve 10 x 10 cm glass aquaria filled with chilled (11°C ± 0.72 S.D), de-chlorinated water to a depth of 8cm. This setup allowed the observer to test 12 fish in one session lasting one hour 15 minutes (including acclimatization). Each tank contained a mirror on one side of the arena so that the fish could see its reflection from anywhere in the tank and each other side was opaque to prevent any external disturbance. An acclimatization zone was included at the opposite end of the arena to the mirror. To avoid any effects due to different tanks, allocation of individuals to tanks was randomised using a random number generator tool on Excel.

Fry were introduced into the acclimatization zone of their assigned tank one by one and left to acclimatize for 15 minutes. After acclimatization the mirror was revealed by raising a partition via a pulley allowing the fish to move around the arena. The fish were observed through a peep-hole in sequential order one at a time for one minute and then the process was repeated. All fish were scored for behaviour

for a total of four minutes over an hour. The observer was unaware of the maternal origin of the fish to eliminate potential observer bias. Behaviours recorded by the observer were the number of dominant postures displayed by the fish (fish swam parallel to the mirror with erect fins, a behaviour believed to enhance assessment of relative size (Johnsson *et al.*, 2003)) and the number of attacks (fish attacked its mirror image as if it were a real opponent). As temperature is known to affect salmonid behaviour (Magoulick and Wilzbach, 1998) this was recorded immediately after the experiment.

Statistical analysis

All analyses were conducted using R 3.0.0. Dominant postures and attacks were summed to generate a measure of total aggression for each individual fish. Data was analysed using generalised linear mixed models (GLMM with Poisson error distribution) using the glmer function from the 'lme4' package (Bates *et al.*, 2012) fitted by Laplace approximation. The full model contained female rearing time (2, 14 and 26 months), timing of emergence (measured as degree days), OBR, egg size category (small/large) and mean batch egg weight (g) as main effects. Fry standard length (mm), degree days of behavioural test and temperature (°C) were also included as covariates. Two-way interactions between egg weight and timing of emergence and OBR and standard length were also included in the model. Random factors were female ID and trap ID nested within female ID. In order to account for over-dispersion, individual ID was also used and a random factor (Bolker *et al.*, 2011).

Measures of OBR were analysed using a linear mixed model with random effect using the lme function from the 'nlme' package (Pinheiro *et al.*, 2007) fitted by Restricted Maximum Likelihood (REML). The full model contained female rearing time (2, 14 and 26 months), timing of emergence, mean batch egg weight (g) and egg size category (small/large) as main effects. Fry standard length (mm), degree day of behavioural test and temperature (°C) were included as covariates. A two-way interaction between egg weight and timing of emergence was also included in the model. Female ID was factored in as a random effect.

All tests were two-tailed with a significance level set to $\alpha = 0.05$. For model selection the step-down protocol was used where non-significant terms (interactions, main effects) were backward eliminated (using Maximum Likelihood (ML) to check for significance for lme). The final, optimal model was refitted using REML following Crawley (2007). Random effects were then tested for significance in the final model and eliminated if not significant. In the results section the t- and P-values are presented for all the fixed factors and interactions in the optimal model (Crawley 2007). All post hoc tests between the female rearing groups were done using the ghlt function from the 'multcomp' package (Hothorn *et al.*, 2008) for multiple comparisons of the mean using Tukey contrasts.

RESULTS

Aggression

A significant effect of female rearing time on aggressive behaviour in newly emerged Atlantic salmon was found (F_2 = 1.77, P = 0.006; Table 2.1). Fry from mothers which had spent less time in the hatchery were more aggressive, after accounting for the effect of egg size, than fry from mothers which had spent longer in captivity (Fig.2.2). Extrapolated over the whole hour of experiment, fry from mothers which had spent 2 months in the hatchery displayed on average 83 aggressive behaviours, whereas those from mothers which had spent 14 and 26 months displayed 71 and 65 aggressive behaviours respectively. Aggression was also positively associated to mean nest egg weight (Table 2.1) and negatively related to emergence time (Table 2.1, Fig. 2.3) and water temperature (Table 2.1). Egg size category, body size, developmental age and OBR had no significant effect on aggressive behaviour and were removed from the model (Appendix I-e). Pairwise comparisons indicated significant differences in offspring behaviour between 2 months and 26 months (z = -2.68, P = 0.020) but no difference between 2 months and 14 months (z = -1.97, P = 0.119) or between 14 months and 26 months (z = -0.87, P = 0.658).

Metabolic rate

OBR was positively associated with egg weight as fry from larger eggs had a significantly higher OBR (Table 2.1). A negative effect of body size and developmental age, measured as cumulative degree days on the date of testing was found (Table 2.1). Female ID accounted for 2.7% of variation in OBR. No significant effect of female rearing time, timing of emergence or egg size category on OBR was found and so was removed from the final model (Appendix I-f).

DISCUSSION

Body size had no effect on aggression in Atlantic salmon alevins in this study, however as the fish were being tested against themselves and were therefore met with an equally sized opponent this is to be expected. However, in dyadic encounters between newly emerged salmonids, alevins with a slight size advantage are able to dominate smaller fish, suggesting that relative body size may influence the outcome of agonistic encounters (Berejikian *et al.*, 1996). Ownership and size have been found to be major predictors of contest success in salmonids competing for territory (Johnsson *et al.*, 1999). Conversely, relative body size of two contestants for a territory has also been found to have no effect on the outcome, which is likely due to the fitness consequences associated with losing a territory (Einum and Fleming, 2000b; Metcalfe *et al.*, 2003).

The ability of territory holders to out compete intruders has generated two main hypothesis. According to the resource-holding power (RHP) hypothesis, residents win because they possess physical or behavioural attributes, for example, large size, strength or aggressiveness, related to fighting ability (Johnsson *et al.*, 1999). Whereas, with the pay-off asymmetry hypothesis, residents win because their payoff from holding a territory increases over time (Johnsson *et al.*, 1999). As the prior ownership effect in salmonids seems to arise from an asymmetry in the value placed on the territory, and fish from mothers which spent longest (26 months) in the hatchery displayed lower levels of aggression, this indicates that they did not perceive their territory as valuable and so did not match their defensive aggression to the perceived quality of the site (Metcalfe *et al.*, 2003). This suggestes that the effect of domestication of the mothers may have led to a reduced ability to establish territories. Additionally, in this study, early emerging fish exhibited higher levels of aggressive behaviour, which if in the wild, might have resulted in competitive displacement and an increase in mortality of late emerging fish (Skoglund *et al.*, 2011). Experimental work on the association between timing of emergence and aggression in Atlantic salmon alevins suggests that early-emerging offspring may have a competitive advantage through prior access to feeding territories (Einum and Fleming, 2000b).

Aggression in newly emerged Atlantic salmon has previously been found to be positively correlated with standard metabolic rate (Cutts *et al.*, 1998). Variation in metabolic rate could therefore underlie life history variation through changes in social status, i.e. individuals with a higher metabolic rate are more likely to be dominant and adopt a faster growth rate than those with low metabolic rate. Ultimately, growth rate can directly affect key life history thresholds in salmonids, such as migration timing and maturation (Wilke *et al.*, 2014). Standard metabolic rate has also been linked to stress coping style as those with a higher metabolic rate tend to exhibit a more active, aggressive life style such as the proactive coping style (Vaz-Serrano *et al.*, 2011).

Metabolic rate and aggression are often positively correlated in Atlantic salmon fry (Cutts *et al.*, 1998; McCarthy, 2001), so the apparent association between timing of first feeding and aggression may actually be a consequence of early-emerging fish having relatively higher metabolic rates (Metcalfe *et al.*, 1995). However, this study found that opercula beat rate (a proxy for metabolic rate) was not associated with aggression whereas early emergence was found to be strongly linked to increased aggression. This finding suggests that there may be an uncoupling of strongly associated behavioural and physiological traits of the proactive stress coping style (Vaz-Serrano *et al.*, 2011). The uncoupling of metabolic rate with aggression and the timing of emergence seen in this study might therefore be a consequence of domestication, due to relaxed selection pressures against maladaptive phenotypes in the hatchery environment (Stringwell *et al.*, 2014), giving rise to increasing behavioural variability (McPhee, 2004). Conversely, a relationship between the time of emergence and stress coping styles has been demonstrated in farmed rainbow trout, *Oncorhynchus mykiss* (Andersson *et al.*, 2013). Therefore, mechanisms other than a relaxation of selection pressures at the timing of emergence may be responsible for the uncoupling of the proactive coping styles seen in this study.

Studies which have considered the differences in aggression between wild born and hatchery-reared salmonid fry have typically found that hatchery fry were more aggressive than their wild counterparts (Swain and Riddell, 1990; Metcalfe *et al.*, 2003). Although this is not consistent as some studies have found no difference between hatchery and wild fish aggressiveness (Johnsson *et al.*, 1996). Our study,

however, does not compare aggression between wild and hatchery-reared individuals - it compares the differences in aggression between hatchery-reared fry from wild mothers which have spent varying amounts of time in the hatchery environment. The environment mothers experience can have an impact on offspring behaviour and salmon fry from hatchery-reared mothers tend to be more aggressive than those from wild females (Swain and Riddell, 1990; Berejikian *et al.*, 1999). Here, alevin aggression was inversely related to time spent in captivity by their mothers. The rearing environment of hatchery broodstock differs considerably to that experienced in the wild, with lack of predators, decreased competition for resources and abundance of food. It is therefore likely that unintentional domestication selection and relaxation of natural selection, due to artificially modified and well-protected rearing environments, are occurring (Araki *et al.*, 2007).

In summary, a negative relationship between aggression and timing of emergence in salmonids was found. However, increased aggression was not related to an increase in metabolic rate which are correlated traits of the proactive stress coping style. This decoupling could be a result of rearing mothers in hatchery conditions, where competition for resources is less severe than in the wild. In other species, offspring originating from high-ranking mothers that provided better nutrition had lower rates of aggression than those from low-ranking mothers (Golla *et al.*, 1999; Nathan *et al.*, 2001; Drake *et al.*, 2008). This suggests that when provisioning is high, aggression may be suppressed as it may not improve fitness. (Ruzzante, 1994; Weber and Fausch, 2003).

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Table 2.1 Results of generalised linear mixed-effects model examining the influences on aggressive behaviour at the time of emergence from the gravel of juvenile Atlantic salmon (n = 299) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on aggressive behaviour (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced. Pairwise comparisons indicated significant differences in behaviour between 2 months and 26 months (z = -2.68, P = 0.020) but no difference between 2 months and 14 months (z = -1.97, P = 0.119) or between 14 months and 26 months (z = -0.87, P = 0.658).

Parameter	**β**	**βSE**	**z**	**p**
Intercept	1.348	0.129	10.476	**<0.001**
14 months	-0.347	0.176	-1.973	**0.048**
26 months	-0.514	0.192	-2.682	**0.007**
Egg Weight	0.171	0.080	2.153	**0.031**
Emergence timing	-0.424	0.073	-5.828	**< 0.001**
Temperature	-0.184	0.074	-2.485	**0.013**

Table 2.2. Results of linear mixed-effects model examining the influences on opercular beat rate (proxy for metabolic rate) at the time of emergence from the gravel of juvenile Atlantic salmon (n = 299) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on OBR (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	62.881	0.620	278	101.422	**< 0.001**
Egg Weight	4.800	0.796	278	6.032	**< 0.001**
Developmental age	-2.475	0.549	278	-4.506	**< 0.001**
Body size (SL)	-3.197	0.760	278	-4.204	**< 0.001**

Figure 2.1.

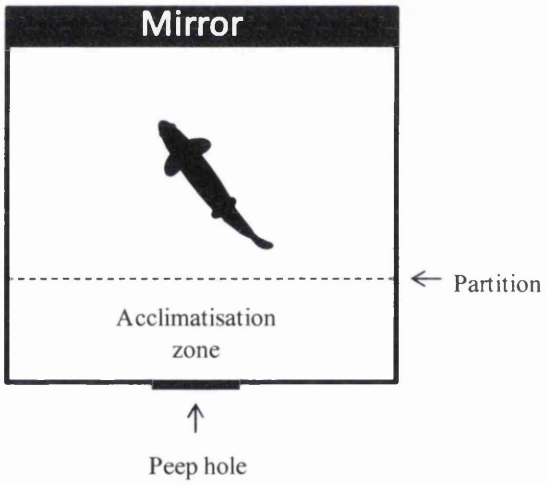

Figure 2.1. Mirror image stimulation (MIS) experimental set-up and schematic diagram of experimental chamber used to assess aggressive behaviour in newly emerged Atlantic salmon fry.

Figure 2.2.

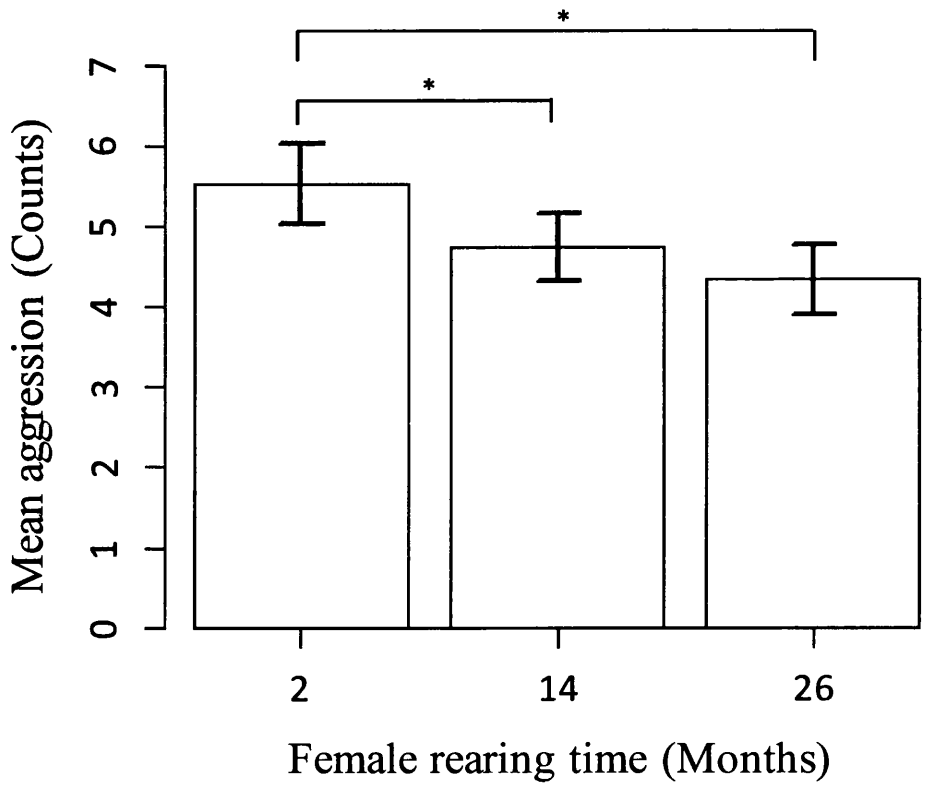

Figure 2.2. Aggressive displays towards mirror image in newly emerged Atlantic salmon fry derived from wild Atlantic salmon females (n = 18) reared for increasing amounts of time in the hatchery environment. Fry were observed for 4 minutes over the course of an hour for dominant postures and attacks towards the mirror. Data shown are mean ± standard error number of aggressive actions from the output of a generalised linear mixed effects model given in Table 2.1. Asterisks denote significant differences ($P < 0.05$) between rearing groups.

Figure 2.3

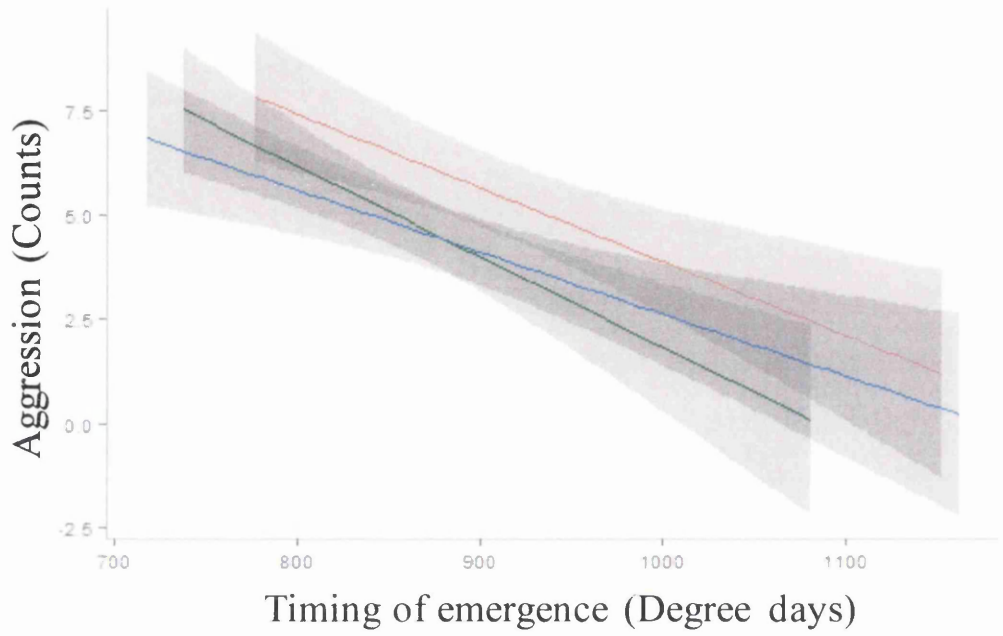

Figure 2.3. Effect of timing of emergence (degree days) on aggressive behaviour (counts) in offspring from wild female Atlantic salmon reared in a hatchery environment for 2 months (Red), 14 months (Green) and 26 months (Blue). Line represents linear mixed-effects model fit of the data. Grey shading represents 95% confidence intervals. Results from linear mixed effects model are given in Table 2.2.

Chapter III

Maladaptation and phenotypic mismatch in hatchery-reared Atlantic salmon released in the wild

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Chapter III

Maladaptation and phenotypic mismatch in hatchery-reared Atlantic salmon *Salmo salar* released in the wild

ABSTRACT

Changes in body shape, fluctuating asymmetry (FA), and crypsis were compared among Atlantic salmon (*Salmo salar*) fry kept as controls in captivity and those released and subsequently recaptured in the wild according to a before-after-control-impact (BACI) design. Hatchery fish that survived in the wild became more cryptic and displayed a much lower incidence of fin erosion and of asymmetric individuals than control fish kept in captivity. Significant differences in body shape were also apparent, and survivors had longer heads, thicker caudal peduncles and a more streamlined body shape than hatchery controls as early as 20 days following stocking, most likely as a result of phenotypic plasticity and non-random, selective mortality of maladapted phenotypes. Hatchery-reared fish typically perform poorly in the wild and the results of this study indicate that this may be due to phenotypic mismatch, i.e. because hatcheries generate fish which are phenotypically mismatched to the natural environment.

INTRODUCTION

Stocking of hatchery-reared juveniles is common practice in many salmon conservation programmes. However the phenotype of fish can diverge greatly in captivity and this may affect post-release survival. The question remains about how long it takes for hatchery fish to adapt to the natural environment and for how long hatchery traits persist in the wild. Rearing animals in captivity, free from predators and with a plentiful supply of food, tends to relax natural selection and this can generate individuals with extreme phenotypes that can persist under favourable conditions, but that would have otherwise perished in the wild (Trut *et al.*, 2009). Indeed, as Darwin first noted (Darwin, 1868), one of the defining traits of domesticated organisms is that they tend to exhibit extreme morphological, behavioural and physiological traits rarely seen under natural conditions (Balon, 2004; Teletchea and Fontaine, 2012). For example, hatchery-reared fish often display extreme growth rates (Saikkonen *et al.*, 2011), aggression levels (Blanchet *et al.*, 2008), risk taking behaviour (Roberts *et al.*, 2011) and predator naivety (Álvarez and Nicieza, 2003) rarely seen among wild fish. Such phenotypic mismatch makes survival of hatchery-reared fish typically low in natural streams (Brown *et al.*, 2003; Jokikokko *et al.*, 2006), and this offers good opportunities for understanding what makes a successful fish: individuals that survive under natural conditions may be expected to be those that are able to adapt most rapidly, or those that resemble wild fish the most (Brown *et al.*, 2003).

Studying adaptive responses in the wild is difficult because the capacity to manipulate phenotypic variation is typically limited (Endler, 1986). However, hatcheries can generate large numbers of individuals, some of which will have extreme phenotypes, and if these are released into the natural environment they will likely be exposed to the same selective pressures as wild fish. Thus, monitoring how hatchery fish with contrasting phenotypes fare in the wild could shed light on the nature of selective forces acting upon juvenile fish in general. Feralisation, i.e. the adaptation of captive-reared animals to natural conditions, may be expected to involve two different processes: (1) selective mortality of maladapted phenotypes (Chittenden *et al.*, 2010), and (2) phenotypic plasticity, i.e. the production of alternative phenotypes in response to environmental change (West-Eberhard, 1989) though their relative roles remain unclear.

High phenotypic plasticity is common in many fish (Smith and Skúlason, 1996), and for some migratory species such as Atlantic salmon (*Salmo salar*) plasticity is probably the consequence of ontogenetic habitat shifts (Von Cramon-Taubadel *et al.*, 2005), which serves to underline the important role that environmental variation has on levels of phenotypic variation of this and other salmonids (Garcia de Leaniz *et al.*, 2007a; Garcia de Leaniz *et al.*, 2007b). For example, body shape variation in juvenile salmonids can be substantial even over small spatial scales, and this is thought to reflect adaptations to local hydrological conditions (Pakkasmaa and Piironen, 2001; Solem and Berg, 2011; Drinan *et al.*, 2012; Stelkens *et al.*, 2012). Indeed, experimentally increasing water velocity tends to produce more streamlined fish (Pakkasmaa and Piironen, 2000). Studies of plasticity in fish have tended to examine phenotypic changes occurring during artificial rearing, and have compared the phenotype of wild and hatchery-reared fish (e.g. Kostow, 2004; Von Cramon-Taubadel *et al.*, 2005); studies addressing changes occurring during adaptation to the natural environment are relatively recent (Rogell *et al.*, 2012; Skaala *et al.*, 2012; Rogell *et al.*, 2013). Comparisons between wild and hatchery fish can reveal divergence due to the effects of artificial selection and domestication (i.e. Fleming and Einum, 1997; Solem *et al.*, 2006), but results are not always easy to interpret because variation in rearing conditions is typically confounded by maternal effects and genetic origin, and what is being compared are essentially different fish (Garcia de Leaniz *et al.*, 2007a).

To better understand the responses of fish to changes in rearing environment, a BACI (Before-After-Control-Impact) design (Manly, 2002) is required, so that phenotypic variation can be partitioned into effects due to the environment and effects due to ontogeny. With this approach, the same group of fish (from the same mothers) is compared before and after they are released into the wild, and the influence of natural vs. artificial conditions can become clearer. Moreover, because survival in hatcheries is typically very high, any phenotypic shifts will be mostly due to phenotypic plasticity, in contrast to natural conditions where changes in trait means will likely be the result of both plasticity and non-random (selective) mortality of some phenotypes. Monitoring changes undergone by hatchery fish in captivity and in the wild, therefore, offers a powerful way of examining the responses of fish to

environmental variation because the differential roles of selection and plasticity can be teased out.

In this study, first generation hatchery-reared juvenile Atlantic salmon from a single population were released into four different river environments while a group was kept at the hatchery to serve as a control. Juveniles were then recaptured twice over their first summer and screened at three phenotypic traits shown previously to be related to fitness in salmonids: morphology (Garcia de Leaniz *et al.*, 2007a; Garcia de Leaniz *et al.*, 2007b), fluctuating asymmetry (i.e. random deviations from perfect bilateral symmetry (Eriksen *et al.*, 2008)), and crypsis (Donnelly and Whoriskey, 1993; Culling *et al.*, 2013). The expectation was that fish released in the wild and subjected to high mortality and large environmental fluctuations would diverge more over time than fish kept under more stable hatchery conditions, which would be affected mostly by phenotypic plasticity. It was also expected that different river environments might select for different phenotypes.

MATERIALS AND METHODS

Origin of fish

Eighteen anadromous Atlantic salmon females (mean fork length 71.3 cm ± 7.3 SD) were crossed with 12 anadromous males (mean fork length 68.3 cm ± 11.0 SD) from the River Taff (South Wales) at the Natural Resources Wales, Cynrig Fish Culture Unit (Brecon, Wales) to produce 36 families according to a 1:2 breeding design (whereby milt from a male was added to half the eggs from a female) on 12th-19th December 2012. Eggs were incubated under standard hatchery conditions on a flow-through system at ambient temperature (5.83 °C ± 1.91 SD). Families were kept separated until first feeding (30th April 2013) and were then distributed evenly into six 2 m^2 tanks (density ~ 1.77 g L^{-1}) and fed at 2.0-3.5% body weight day^{-1} under natural photoperiod until late June 2013.

Experimental releases

On 25th June 2013, Atlantic salmon 0+ fry were accurately hand counted into four groups of 15,000 fish each and transferred into four separate tanks (one per stocking site) to produce 60,000 fish in total. Fish were released along 50 metre sections of four first order stream sites on the headwaters of the river Taff between 27th June and 1st July. Experimental release sites were selected based on the absence of salmon spawning due to impassable barriers and their location along a altitudinal gradient (from 280 m to 153 m above sea level) to maximize environmental variation: Rhondda Fach at Maerdy, River Clydach at St Gwyno Forest, River Dare at Aberdare and River Cynon at Penderyn (Table 3.1). At each site, pH, water temperature (°C), river width (m), water depth (cm), dominant substrate diameter (mm), water velocity (cm s^{-1}) and extent of vegetation cover (%) were recorded along three evenly spaced transects, one at the downstream end, one midstream and one at the upstream end. Sightings (or markings) of three common fish predators (grey heron, *Ardea cinerea* L. 1758; common kingfisher, *Alcedo atthis* L. 1758; Eurasian otter, *Lutra lutra* L. 1758) were also noted at the time of stocking and at each recapture time to provide an index of predation pressure. As a control group, 300 fish from the same batch of fish were brought to a recirculation system at Swansea University on the day of stocking (time 0), where they were kept under

standard hatchery conditions in three 0.65 m diameter x 0.85 m depth circular tanks (density ~ 0.22 g L^{-1}) and fed 2.5% body weight day^{-1} on commercial fish food under a 14L:10D photoperiod.

Recapture of stocked fish in the field

At each of the four stocking sites, fish were sampled along 6 x 50 m stations (distributed evenly throughout the whole length of the site) using semi-quantitative point electro-fishing carried out from bank to bank in a zigzag fashion to cover all micro-habitats. Sampling was carried out at 20 days post-release (DPR) [15th - 18th July - Time 1] and again at 55 DPR [19th - 22nd August - Time 2], and these were compared to the control group kept at the hatchery to conform to a BACI design (Fig. 3.1). In most cases, 100 salmon fry were sampled per site, except at Maerdy at Time 2 where only 70 fry could be recaptured due to high water. In each case, salmon recaptures were transported live to the salmon laboratory at Swansea University where they were held in a tank for 24 hours to standardize variation in gastric content that could affect measurements of body shape. Measures of crypsis were taken first and the fish were then humanely killed by an overdose of anaesthesia according to Home Office schedule one. Brown trout (*Salmo trutta*, L. 1758) and other fish species caught during field sampling were counted to provide an index of inter-specific competition and returned live to the river. To provide a reference baseline for the body shape of wild fish, 18 Atlantic salmon 0+ fry from the same approximate age (but not derived from stocking) were captured by electro-fishing in a tributary in the lower part of the River Taff system (river Rhondda, grid reference ST064903) on 27th July.

Morphometric analysis

A sample of 90 hatchery fish were randomly selected at the time of stocking to serve as a baseline [time 0]. Subsequently, 30 fish from each of the four stocking sites and 30 fish from the hatchery control group (150 in total) were sampled at each time period (time 1, time 2). For morphometric analysis, fish were photographed (Canon EOS 400D, www.canon.com; 90 mm TAMRON SP Di 1:1 macro, www.tamron.eu/uk) from a fixed distance, facing left and with their fins extended, against a standard background fitted with a scale bar. For each specimen, 19

landmarks used in previous studies (Blanchet *et al.*, 2008; Pulcini *et al.*, 2013) were digitized using the tpsDig 2.16 software (Rohlf, 2004). To correct for possible bias due to body bending, the unbend application of the tpsUtil programme (Rohlf, 2010) was employed, using three additional landmarks along the lateral line to generate corrected landmark coordinates (Haas *et al.*, 2010). Co-ordinates were then imported into the software MorphoJ for procrustes superimposition (Klingenberg, 2011), which computes an average shape to which specimens are aligned in order to remove the effect of size from the study of morphological variation (Vehanen and Huusko, 2011).

Principal component analysis (PCA) was carried out on the covariance matrix followed by separate two-way ANOVAs on the first two PCA scores to assess the effects of rearing environment (field vs. hatchery control) and time on the major features of body shape variation. Phenotypic trajectories of hatchery controls and fish recaptured in the wild were generated by calculating temporal changes in mean PC1 and PC2 along with their 95 CI (Adams and Collyer, 2009). Following PCA, discriminant function analysis (DFA) was carried out to quantify the ability to discriminate between hatchery controls and field recaptures at each time point; cross-classification reliability was assessed by using the leave-one-out procedure, and visualized by plots of canonical variate scores at each site and time period. To assess variation in pectoral fin length, pectoral fins were digitized separately using ImageJ (Abràmoff *et al.*, 2004) and analyzed via ANCOVA with fork length as a covariate in log10-transformed values. Opercular and caudal fin erosion were visually assessed on a scale from 0 (nil) to 3 (completely eroded) according to Roberts *et al.* (2011), and comparisons assessed via the Mann-Whitney or Kruskal-Wallis test. The observer was blind to the origin of fish when scoring erosion levels, which have been found to be highly repeatable (Hoyle *et al.*, 2007). Statistical analyses were carried in R 3.0.0.

Variation in crypsis

To quantify variation in crypsis, fish were first placed in individual 25 L white buckets filled to approximately 10 cm with aerated water and covered with a lid. After 10 minutes in the white bucket, a photograph ('white photo') was taken of each fish against a standard, low reflectance grey background fitted with a Tiffen Q-13

colour separation guide (www.tiffen.com) and a scale bar, using the same camera and settings as for the morphometric measurements described above. Fry were then transferred to 25 L aerated black buckets, held for another 10 minutes, and a second photograph ('black' photo) taken as above. Reflectance values were obtained from each pair of fish photographs (white vs. black) along three points on each of the three central parr marks of the fish and their corresponding flanks using Image-J, following the procedure described in Culling *et al.* (2013). Gray scale calibration was achieved by taking three readings from the white and black Tiffen Q-13 reference colours, and these were then used to derive standardized reflectance values for each fish. Parr mark contrast was defined as the difference between the readings on the parr marks and the flanks, and a crypsis index was calculated as the difference in parr mark contrast between the black and the white photographs taken on the same fish. Two-way ANOVAs were used to test for variation in crypsis index and parr mark contrast with sampling period and fish origin as fixed factors; for parr mark contrast separate tests were carried out for photographs against white and black backgrounds to avoid pseudoreplication.

Fluctuating asymmetry

Fluctuating asymmetry (FA) was assessed in relation to three bilateral meristic structures fixed in formaldehyde and viewed under an Olympus SZ40 stereo microscope (www.olympus.co.uk) at 4x magnification: (1) number of gill rakers in the upper and lower sections of the first gill arch, (2) number of rays in the pectoral fins, and (3) number of rays in the pelvic fins. Fin ray counts were recorded disregarding any branching, scoring only the base of each ray. To test the reliability of the FA scoring, 30 fish were selected with the help of random number generator and meristic counts on each structure were carried out twice in a blind fashion. Repeatability was calculated as the agreement intra-class correlation coefficient (ICC) with the 'psy' R-package, defined as the ratio of the subject variance divided by the sum of the subject variance, the observer variance and the residual variance (Wolak *et al.*, 2012). The proportion of asymmetric individuals for at least one trait was analysed in relation to sampling period and origin of fish as fixed factors by a generalized linear model with a binomial or quasibinomial error structure using R 3.0.0 as per Crawley (2007). The apparent relative mortality of asymmetrical fish in

the wild was determined by calculating the proportion of asymmetrical fish that must have died (or emigrated) from the population compared to symmetrical fish which was taken as a baseline equal to one.

RESULTS

Phenotypic trajectories and body shape divergence

Analysis of phenotypic trajectories via PCA plots revealed a marked effect of rearing environment on the body shape of juvenile salmon, resulting in increasing phenotypic divergence of fish in the wild compared to control fish held at the hatchery (Fig. 3.2). Ontogenetic changes in body shape in the hatchery environment occur mostly along PC2 and result in fish with shorter heads and deeper bodies, while changes in body shape in the natural environment occur mostly along PC1 and result in fish with more streamlined bodies and thicker caudal peduncles. Results of ANOVA on PC scores confirm that body shape changes significantly with both time (PC1 - $F_{1,333} = 21.51$, $P < 0.001$; PC2 - $F_{1,333} = 18.82$, $P < 0.001$) and rearing environment (PC1 - $F_{1,333} = 23.19$, $P < 0.001$; PC2 - $F_{1,333} = 63.78$, $P < 0.001$); a significant time x rearing environment interaction was found for PC2 ($F_{1,333} = 22.87$ $P < 0.001$) but not for PC1 ($F_{1,333} = 1.29$, $P = 0.258$).

Body shape discrimination

Results of discriminant function analysis (DFA) are highly significant for all pairwise body shape comparisons (Fig. 3.3), and reveal a high discrimination in body shape between hatchery controls and field recaptures (93-97%), as well as between fish sampled at different time periods (87-95%), confirming the results of PC ANOVA. DFA comparisons also indicate that differences in body shape provide good discrimination not only between hatchery controls and wild fish (84%, Hotellings $T^2 = 805.6$, $P < 0.001$), but also between wild and stocked fish (100%, Hotellings $T^2 = 998.01$, $P < 0.001$). In general, compared to initial baseline values at stocking time, fish kept in the hatchery develop deeper bodies, shorter heads, and shorter caudal peduncles over time, whereas almost exactly the opposite occurs when they are released in the wild.

Variation in body shape among release sites

Plots of the first two canonical variate scores (CV1-CV2) clearly separate hatchery fish from fish released in the wild, and to a lesser extent, also serve to identify fish recaptured in different field sites on the basis of their body shape (Fig. 3.4). All

pairwise DFA comparisons of body shape were significantly different among release sites at $P<0.01$, except between Maerdy and Clydach (first recapture T1, $P = 0.738$; second recapture T2, $P = 0.162$), with fish stocked in the River Cynon at Penderyn being the ones most different from the rest (Fig 3.4).

Fin and opercula erosion

Compared to hatchery controls, fish recaptured in the wild had significantly less erosion in the caudal fin (Mann-Whitney, $P < 0.001$) and the operculum ($P < 0.001$) on both sampling occasions (Table 3.2). Also, unlike in the hatchery, where fish showed no change in caudal fin erosion ($P = 0.056$) or even increased their opercular erosion ($P = 0.006$), erosion among stocked fish decreased significantly with time spent in the wild ($P <0.001$). The length of the pectoral fins did not differ significantly between hatchery controls and field recaptures while statistically controlling for variation in body size (ANCOVA $F_{1,276} = 3.48$, $P = 0.063$).

Crypsis

Following stocking, parr mark contrast decreased significantly between day 20 and day 55 for both types of fish (time effect; white background $F_{1,92} = 12.827$, $P = 0.001$; black background $F_{1,92} = 20.013$, $P < 0.001$) and was always much higher for field recaptures than for hatchery controls, regardless of background colour (origin effect; white background $F_{1,92} = 16.000$, $P < 0.001$; black background $F_{1,92} = 22.735$, $P < 0.001$), interactions being non-significant in both cases ($P > 0.1$; Fig 3.5). In contrast, variation in crypsis index (i.e. the change in parr mark contrast when fish were moved from the white to the black background) did not change between sampling periods ($F_{1,92} = 1.250$, $P = 0.266$) or differed significantly between hatchery controls and field recaptures ($F_{1,92} = 1.076$, $P = 0.302$), the interaction being non-significant ($F_{1,92} = 3.400$, $P = 0.068$).

Fluctuating asymmetry and relative survival of asymmetric fish

Meristic counts on duplicate samples were highly repeatable, as indicated by the very high intra-class correlation coefficients (ICC pectoral fin rays 1.000; pelvic fin rays 0.998, 95CI = 0.954-1.000; gill rakers 0.988, 95CI = 0.976-0.997). Gill raker number

was the trait with the highest proportion of asymmetrical individuals, followed by number of pectoral fin rays and number of pelvic fin rays (Table 3.3). Most of the fish kept in the hatchery (116/134 or 86%) were asymmetrical for at least one of the three meristic traits examined and this proportion remained unchanged over time (Fig. 3.6a, Table 3.3). In contrast, the proportion of asymmetrical fish in the wild decreased sharply after stocking, and by 55 days post-release only 29.9% of individuals (35 out of 117) were found to be asymmetrical (binomial 95CI on proportions = 0.218-0.391). Analysis by generalized linear models with binomial errors revealed a significant effect of rearing environment (deviance $G^2_1 = 29.19$, $P < 0.001$) and time (deviance $G^2_2 = 51.09$, $P < 0.001$) on the proportion of asymmetrical individuals, as well as a significant interaction time x rearing environment (deviance $G^2_2 = 17.37$, $P < 0.001$). Given that there was no mortality among hatchery controls over the period of study and that any variation in asymmetry at the hatchery could only be due to sampling error, it was possible to estimate the apparent relative survival of asymmetrical individuals in the wild in relation to that of symmetrical ones. The results (Fig 3.6b) indicate that the relative survival of asymmetrical fish was 53% of the survival of symmetrical fish 20 days after release (binomial 95CI = 43.3-63.6) and dropped to only 8.5% at 55 days post-release (binomial 95CI = 3.6-15.4).

DISCUSSION

This study employed a BACI approach to investigate how the morphology, crypsis and fluctuating asymmetry of juvenile Atlantic salmon change when hatchery-reared fish are released into the wild, providing in this way an assessment of the process of fish feralisation, i.e. the adaptation of fish to the natural environment or the process of domestication in reverse (Price, 2002; Zeder, 2012).

The phenotype of salmon fry changed substantially over time, and fish in the wild diverged significantly from hatchery fish as early as 20 days post-release. Compared to hatchery controls, juvenile salmon in the wild became more streamlined, more symmetrical, developed longer heads, thicker caudal peduncles, and their caudal fins and opercula regenerated. The fitness implications of such phenotypic changes are difficult to predict in the wild but are likely to be adaptive because morphology affects swimming efficiency (Pakkasmaa and Piironen, 2000; Pakkasmaa and Piironen, 2001), feeding ability (Adams *et al.*, 2003), and predator avoidance (Drinan *et al.*, 2012). For example, streamlining of body shape and head length has been demonstrated in salmonids reared in fast water (Pakkasmaa and Piironen, 2000) and is thought to reduce drag and swimming costs (Enders *et al.*, 2004), making foraging more energetically efficient (Pakkasmaa and Piironen, 2001; Vehanen and Huusko, 2011; Drinan *et al.*, 2012). Head length, body depth and fin size are the characters that best discriminate among juvenile salmon from different rivers in Norway (Solem and Berg, 2011) and variation in these are thought to reflect adaptations to local conditions in Atlantic salmon (Garcia de Leaniz *et al.*, 2007a; Garcia de Leaniz *et al.*, 2007b). Confinement in hatchery tanks with low water velocity and plentiful food increases fat deposition and results in deepening of the body amongst hatchery–reared salmonids (Pulcini *et al.*, 2013), and this was also evident in our study. Similar changes have been reported for other fish species and serve to highlight the different selective pressures that fish experience in natural and artificial environments (Lorenzen *et al.*, 2012), and the strong effects that food regime and swimming activity can have on fish body shape (Pakkasmaa and Piironen, 2000; Marcil *et al.*, 2006).

Pectoral fins are important for station holding in juvenile Atlantic salmon as they act as hydrofoils, generating downward force and allowing fish to occupy high velocity feeding stations (Armstrong *et al.*, 2003; Drinan *et al.*, 2012). Fin and

opercular erosion are a common problem in hatchery-reared salmonids (Bosakowski and Wagner, 1994; Latremouille, 2003) which tend to have shorter fins than wild fish (Blanchet *et al.*, 2008). This was also the case in this study with respect to opercular and caudal fin erosion, which may have affected the swimming ability of stocked fish, though no difference was found for pectoral fin length. The fact that erosion decreased with time in the wild, but increased in the hatchery, probably reflects some regeneration under natural conditions but is also consistent with selection against maladapted phenotypes, in this case against fish with shorter than average tails and shorter than average opercula. The latter is also suggested by changes in body shape, which revealed an enlargement of head length in the wild and a shortening in the hatchery, likely as a result of opercular erosion.

Fry in the wild also displayed darker parr marks than hatchery controls, and this would have made them more cryptic and less conspicuous to predators (Donnelly and Dill, 1984; Donnelly, 1985; Donnelly and Whoriskey, 1993; Culling *et al.*, 2013). Salmonids show considerable plasticity in parr mark pigmentation that depends on diet, but also responds to a number of environmental variables including water transparency and substrate type, which is likely to be under selection (Culling *et al.*, 2013). Colour change in salmonids can occur rapidly (Westley *et al.*, 2013) and this study found significant differences in parr mark contrast within just 20 days. The low parr mark contrast displayed by hatchery controls is typical of slow flow, low gradient environments (i.e. pools) with homogenous backgrounds (Donnelly and Dill, 1984), which characterise hatchery tanks. Moving fish from a light to a dark background had no consistent effect on the crypsis index in our study, which was unrelated to sampling period or fish origin. The ability to change colour instantly, termed physiological colour change (Westley *et al.*, 2013), may require longer acclimatization periods than the 10 min used in our study. This is similar to the findings of Donnelly & Whoriskey (1993) who reported that juvenile Atlantic salmon acclimatised to a light background were unable to camouflage to a darker background in order to avoid predation.

Under stabilizing selection, feralisation may be expected to result in phenotypic convergence by selecting some optima on behaviours and body plan (Zeder, 2012), but this was not the case in our study. Juvenile Atlantic salmon stocked in the wild strongly diverged from hatchery fish with increasing time spent

in the wild but did not appear to converge towards the body shape of wild fish, which remained morphologically differentiated from both hatchery controls and stocked fish. Although our sample of wild fish is admittedly small, these results serve to highlight that morphometric comparisons also need to consider potential differences in genetic background, that phenotypic variation in juvenile Atlantic salmon is probably the norm, and that there may not be just one single body plan optimal for all environments. Fish released at different sites had statistically different (albeit only slightly) body shapes at recapture, and recaptured fish were not more similar to wild fish than hatchery fish were. There was some phenotypic divergence among the four field sites and some evidence that these may have been related to the environment fish lived in. Thus, fish released at Penderyn developed the most distinct body shape. As this site is characterised by having the slowest water velocity, the deepest water, the smallest substrate and the greatest extent of pool habitat, it is tempting to speculate on a relationship between river habitat and body shape. Atlantic salmon fry prefer shallow riffles with water velocity between 20 and 40 cm s^{-1} and avoid slow flowing waters with velocities less than 5-15 cm s^{-1} (Armstrong *et al.*, 2003). Penderyn had average velocity of 18 cm s^{-1}, so it may not have been an ideal habitat for Atlantic salmon fry. Studies in other species have found that fish can respond rapidly to slow flows by developing deeper bodies and smaller heads (Haas *et al.*, 2010) and this may have also been the case at Penderyn.

Taken together, the phenotypic shifts observed among Atlantic salmon fry in the wild are likely to be adaptive because the traits involved are related to fitness in salmonids (Garcia de Leaniz *et al.*, 2007a; Garcia de Leaniz *et al.*, 2007b), and the nature of the changes were in the expected direction. Yet, the extent to which these were the result of phenotypic plasticity or non-random mortality (or emigration) of maladapted phenotypes is unclear. Given that there was no mortality in the hatchery, two different mechanisms must have been at work: phenotypic plasticity in the hatchery, and plasticity plus selection in the wild. Consideration of fluctuating asymmetry as an index of development instability, i.e. the inability by an embryo to produce a consistent phenotype in a given environment (Johnson *et al.*, 2004), may shed some light on the relative importance of plasticity and selection. The development of bilateral structures on opposites sides of an organism (such as pectoral fins) is controlled by the same genes, and any deviations from perfect

bilateral symmetry are thought to result from environmental and genetic stressors (Johnson *et al.*, 2004). High levels of fluctuating asymmetry in some hatchery stocks (Vollestad and Hindar, 1997; Yurtseva *et al.*, 2010) have been linked to maternal stress, environmental fluctuations during embryo development, and reduced genetic variation (Leary *et al.*, 1985b; Leary *et al.*, 1985a), though a general relationship linking FA and heterozygosity appears only weak (Vøllestad *et al.*, 1999). In this study, the proportion of asymmetrical individuals remained high at 86% in the hatchery and did not change over time, but decreased sharply in the wild, and by day 55 only 30% of field recaptures were asymmetrical. As the meristic structures considered are not plastic but become fixed instead during early development (Swain and Foote, 1999; Yurtseva *et al.*, 2010), the observed decrease in the frequency of asymmetrical individuals in the wild must have been due to a higher mortality (or emigration) of asymmetrical fish relative to symmetrical ones. To generate the observed results, it was estimated that asymmetrical fish must have been c. 12 times more likely to die or emigrate from the study area than symmetrical fish. Given that the frequency of asymmetrical individuals can be up to four times higher among hatchery fish than among wild fish (Crozier, 1997; Moran *et al.*, 1997; Vollestad and Hindar, 1997), much of the phenotypic changes in the present study must therefore be attributed to non-random mortality (or emigration) of maladapted hatchery phenotypes, and not simply to plasticity.

A significant decrease in fluctuating asymmetry with time has been reported previously for wild Atlantic salmon by (Moran *et al.*, 1997), who noted that such changes did not occur in captivity, and who suggested a role for natural selection in the purging of asymmetrical individuals from wild populations. Several authors have also found a positive association between FA and environmental stress in fishes (reviewed by Allenbach, 2011), as well as a decrease of FA with fish age, which is suggestive of non-random mortality of asymmetrical fish and, therefore, of selection (Sanchez-Galan *et al.*, 1998). Comparison of different meristic structures indicates that the highest incidence of asymmetrical individuals was found for the number of gill rakers, followed by number of pectoral fin rays, and by the number of pelvic fin rays, in agreement with previous studies on Atlantic salmon (Crozier, 1997). In general, field recaptures were 2-4 times more symmetrical than hatchery controls, depending on the structure, but the extent to which FA for individual traits can be

related to their effect on fitness remains unclear (Moran *et al.*, 1997; Vollestad and Hindar, 1997).

A link between form and function is assumed to exist in the body shape of fishes (Thompson, 1961), and natural selection may be expected to favour those phenotypes that increase fitness in local environments (Solem *et al.*, 2006). Hatchery-reared fish typically perform poorly in the wild (Munakata *et al.*, 2000; Jokikokko *et al.*, 2006) and the results of our study suggest that this may be due to phenotypic mismatch, i.e. because hatcheries generate fish which are phenotypically mismatched to the natural environment.

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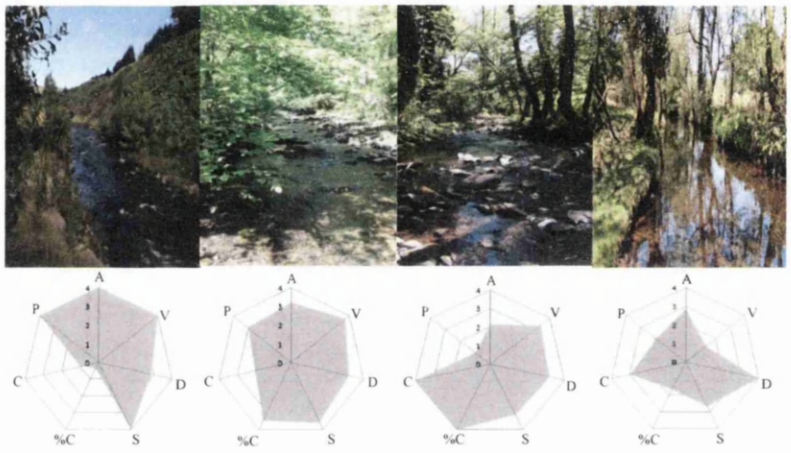
Table 3.1. Abiotic and biotic characteristics of the four stocking sites on the River Taff, South Wales. Competition and predation were ranked from Low to High based on the relative abundance of 0+ salmon fry and sightings of aquatic predators relative to the average for the four sites. Sun-ray plots show environmental profiles of each site based on seven variables standardised from 0 to 4 (A = Altitude (m), V = Velocity (m s^{-1}), D = Depth (cm), S = Substrate size (cm), % C = Cover, C = Competition, P = Predation).



Variable/Site	Maerdy	Clydach	Aberdare	Penderyn
GPS coordinates	SS971993	ST044968	SN991025	SN953065
Area (m^2)	6792	6315	5987	5958
Altitude (m)	280	224	153	202
Width (m)	6.86	6.76	5.07	5.70
Water velocity (m s^{-1})	0.52	0.49	0.44	0.18
Temperature (°C)	14.7	15.7	16.7	15.3
pH	6.44	6.45	6.40	6.33
Depth (cm)	16.2	18.4	18.2	22.7
Substrate size (cm)	16.3	14.7	12.6	10.9
Canopy cover (0-3)	0.22	2.11	2.44	1.00
Competition	Low	Intermediate	High	High
Aquatic predation	High	Intermediate	Low	Low
Avian predators seen	heron, kingfisher	None	none	heron, kingfisher
Terrestrial predators	none	None	otter	none
Stocked previous year	yes	Yes	no	yes
CPUE T1-T2 (0+/m^2)	0.0249-0.0109	0.0238-0.0250	0.0317-0.0249	0.0737-0.0413

CPUE = Catch per unit effort. Calculated as number of 0+ salmon fry caught per unit area.

Table 3.2. Mean (± S.E) scores of caudal fin and opercular erosion of hatchery controls and field recaptures at various times since stocking, associated non-parametric Mann-Whitney W statistic and significance values. Significant pairwise comparisons are indicated in bold.

Trait/Sampling event	Hatchery Controls	Field Recaptures	W	*P*
Caudal fin erosion				
Time 0 – Stocking	0.39 (± 0.096)	0.41 (± 0.088)	718.0	0.785
Time 1 – 20 days	0.78 (± 0.145)	0.18 (± 0.044)	2286.5	**< 0.001**
Time 2 – 55 days	0.57 (± 0.092)	0.10 (± 0.034)	2409.5	**< 0.001**
Opercular erosion				
Time 0 – Stocking	0.50 (± 0.098)	0.51 (± 0.089)	722.0	0.830
Time 1 – 20 days	0.93 (± 0.091)	0.39 (± 0.055)	2293.5	**< 0.001**
Time 2 – 55 days	0.63 (± 0.089)	0.09 (± 0.028)	2520.5	**< 0.001**

Table 3.3. Proportion of asymmetric individuals for three meristic traits (95 binomial CI) at various sampling times.

Trait/Group	Baseline - T_0	T_1- 20 days	T_2 - 55 days
Pectoral fin rays			
Hatchery Controls	0.57 (0.41 - 0.71)	0.31 (0.11 - 0.58)	0.29 (0.10 - 0.55)
Field Recaptures	0.60 (0.43 - 0.75)	0.28 (0.18 - 0.39)	0.07 (0.02 - 0.15)
Pelvic fin rays			
Hatchery Controls	0.48 (0.30 - 0.67)	0.22 (0.09 - 0.42)	0.21 (0.08 - 0.41)
Field Recaptures	0.49 (0.32 - 0.65)	0.30 (0.22 - 0.40)	0.10 (0.05 - 0.17)
Gill rakers			
Hatchery Controls	0.82 (0.68 - 0.92)	0.67 (0.47 - 0.83)	0.48 (0.30 - 0.68)
Field Recaptures	0.72 (0.56 - 0.85)	0.59 (0.50 - 0.68)	0.14 (0.09 - 0.22)

Figure 3.1.

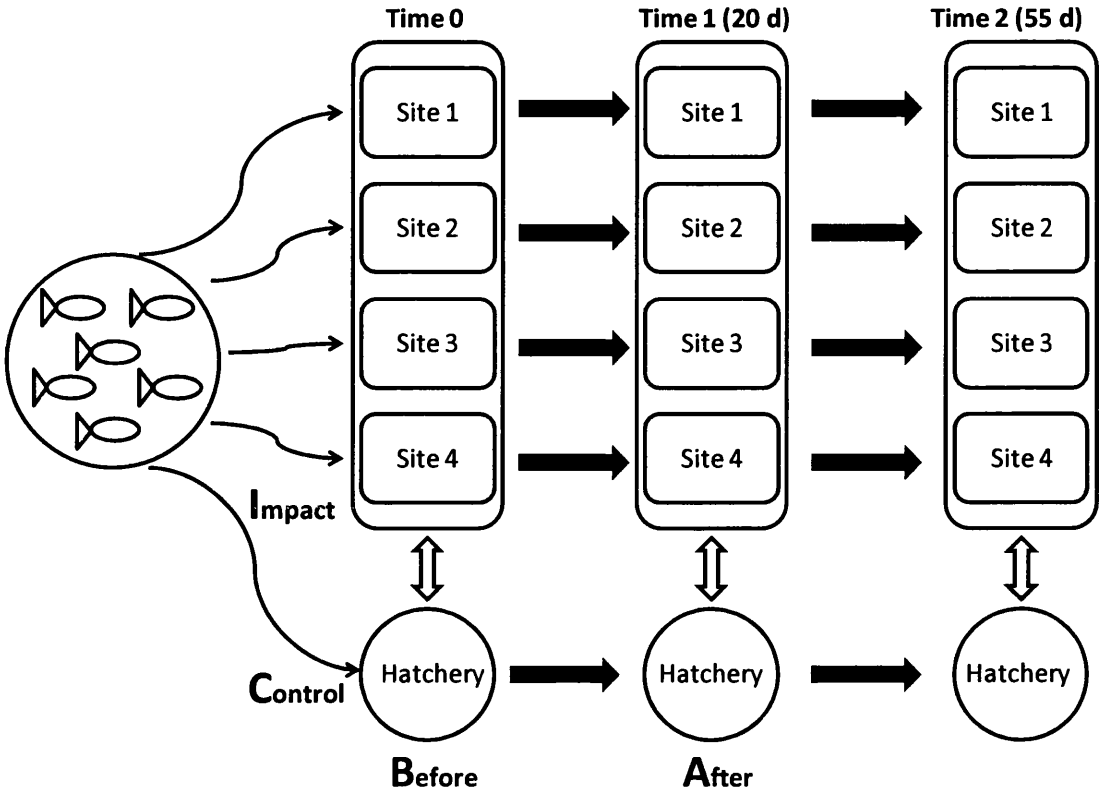


Figure 3.1. Before-after-control-impact (BACI) design employed to examine phenotypic shifts undergone by hatchery-reared juvenile Atlantic salmon released into the natural environment. Hatchery fish (Control) were stocked into four sites in the wild (Impact) and comparisons made Before and After release.

Figure 3.2

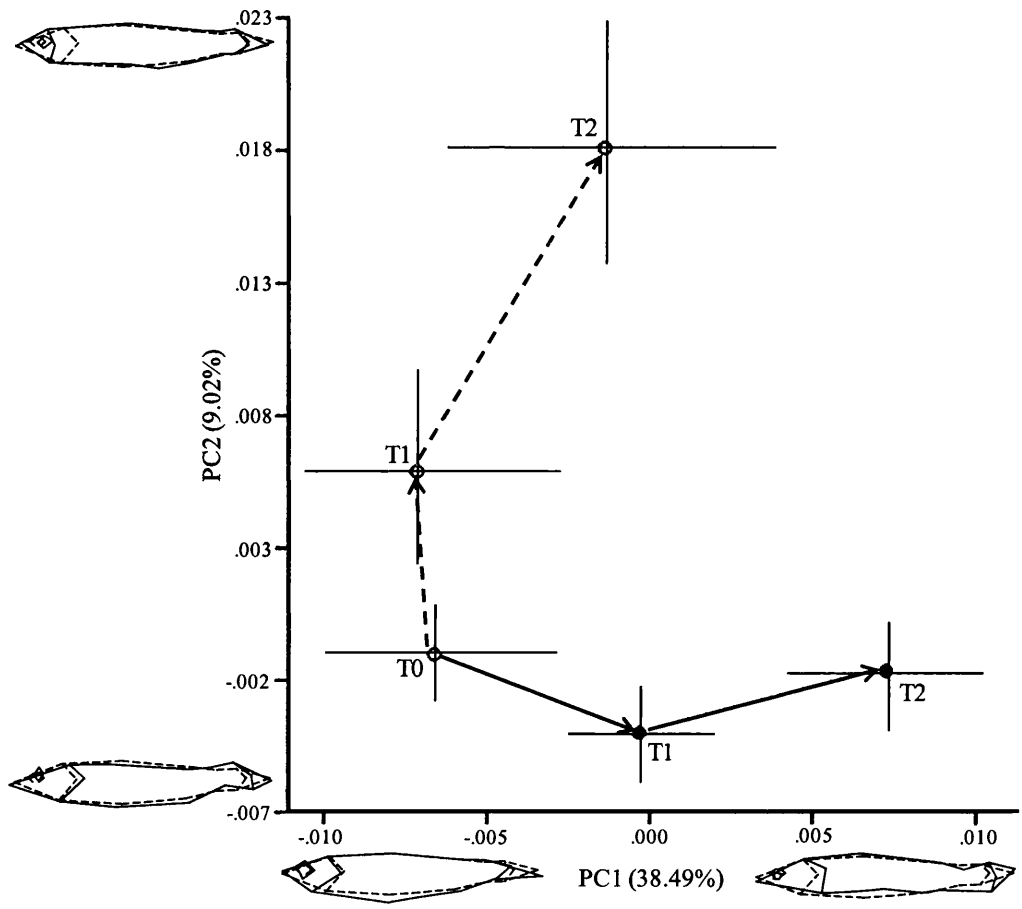


Figure 3.2. Phenotypic trajectories in body shape of juvenile Atlantic salmon held at a hatchery as controls (○) or released in the wild (●). Depicted are the means of the first two principal components (± 95% CI) at three sampling times (T0, T1, T2) during the first two months of the first growing season (July-August). Shape variation along each PC is shown by their relative splines at x2 magnification (—) in comparison to the average body shape (- - -).

Figure 3.3

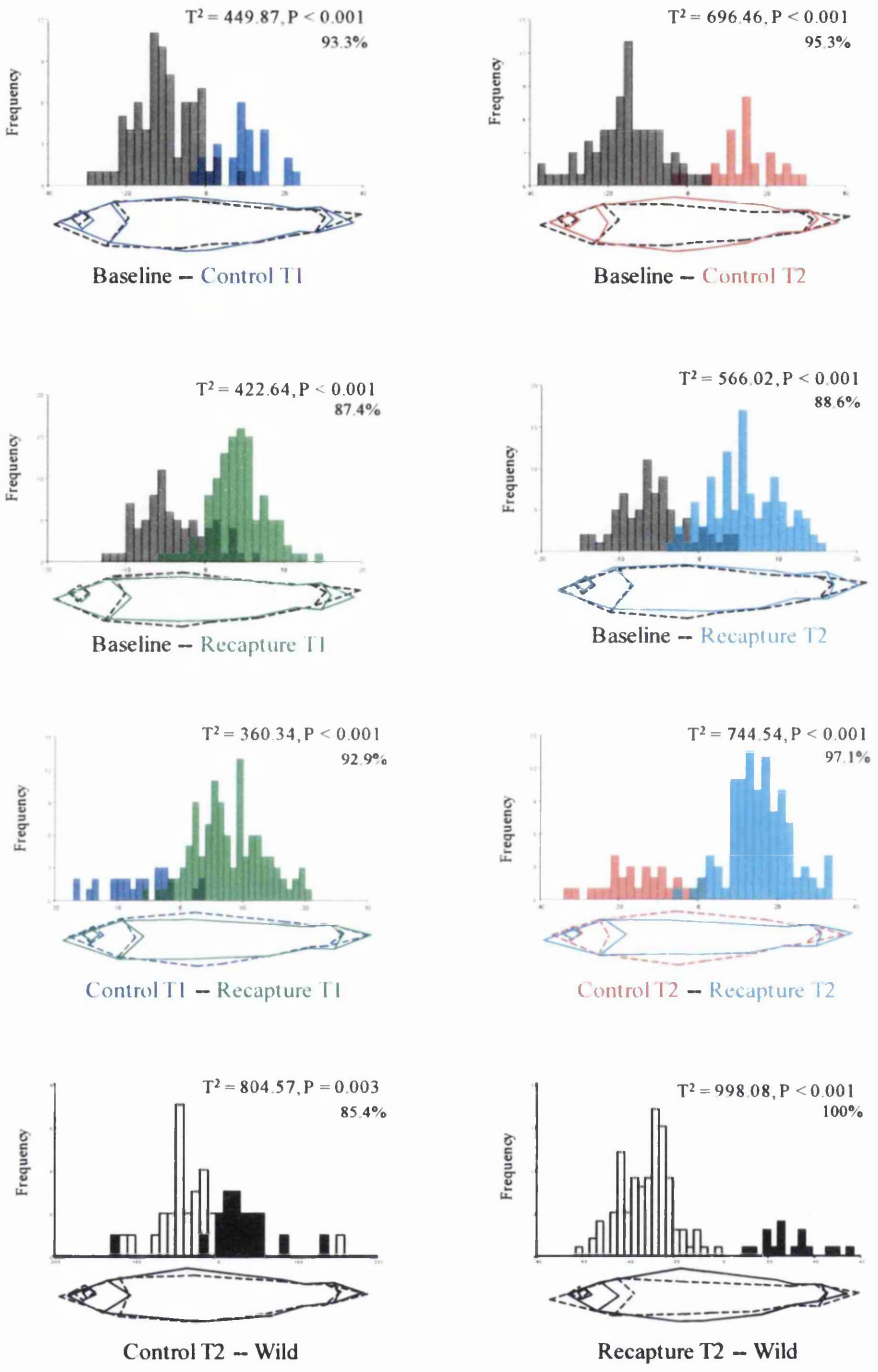


Figure 3.3. Discriminant function scores of pairwise comparisons in body shape of juvenile Atlantic salmon, showing leave-one–out % correct classification, Bonferroni-adjusted probabilities associated with Hotellings T^2, and relative splines (x3 magnification) of body shape change (—) in comparison to the reference shape (- - -).

Figure 3.4

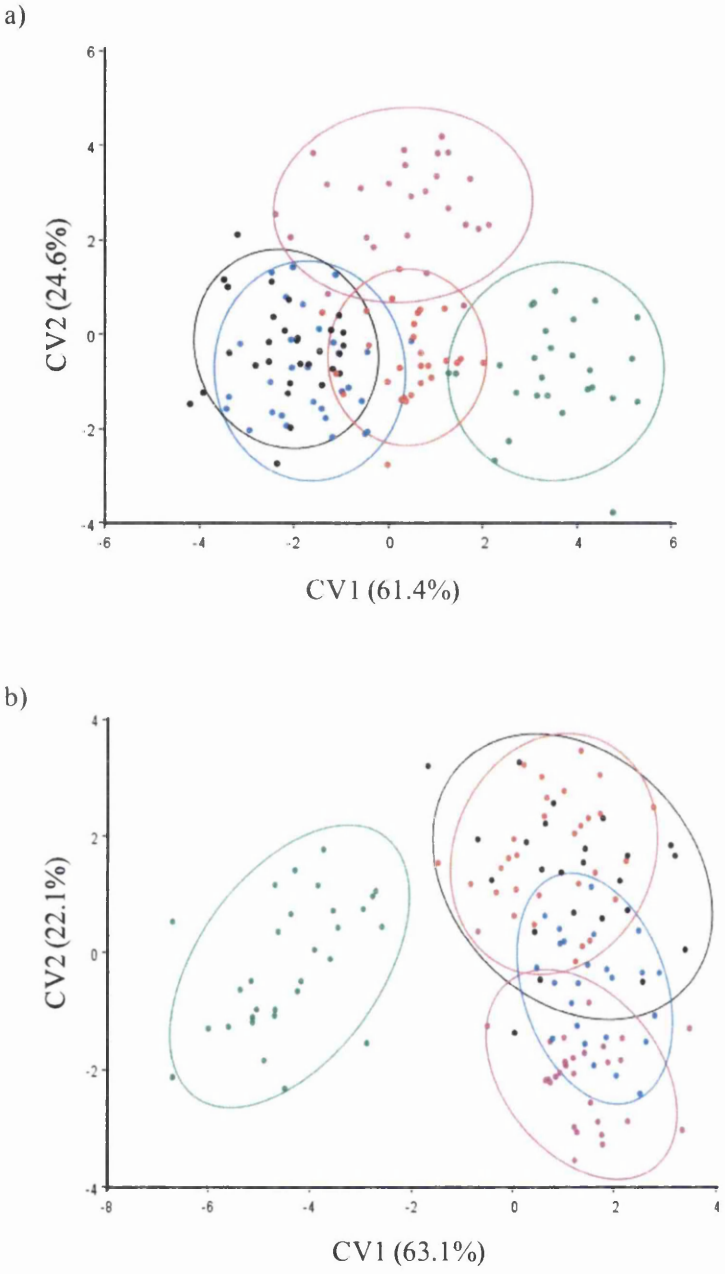


Figure 3.4. Canonical variate plots showing morphometric separation of juvenile Atlantic salmon released at four sites in relation to hatchery controls at (a) 20 days post stocking, and (b) 55 days post stocking. Hatchery Controls (●), Aberdare (●), Clydach (●), Maerdy (●), and Penderyn (●).

Figure 3.5

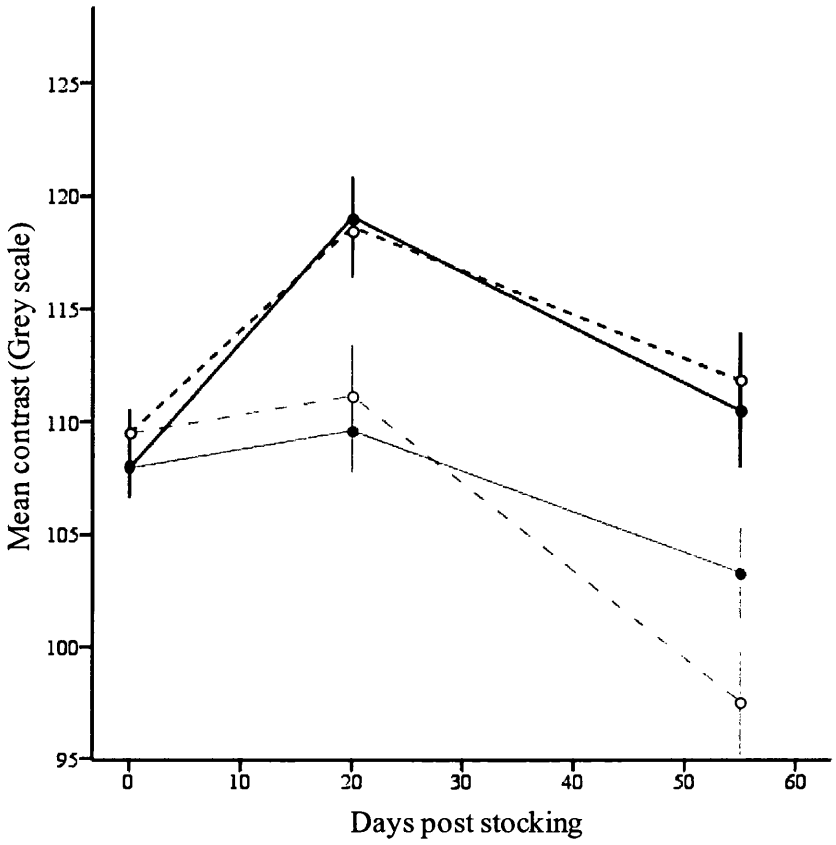


Figure 3.5. Variation in mean parr mark contrast (± 1 S.E) of hatchery controls (•) and field recaptures (•) after being kept for 10 minutes in a white (—) or black (- - -) container .

Figure 3.6

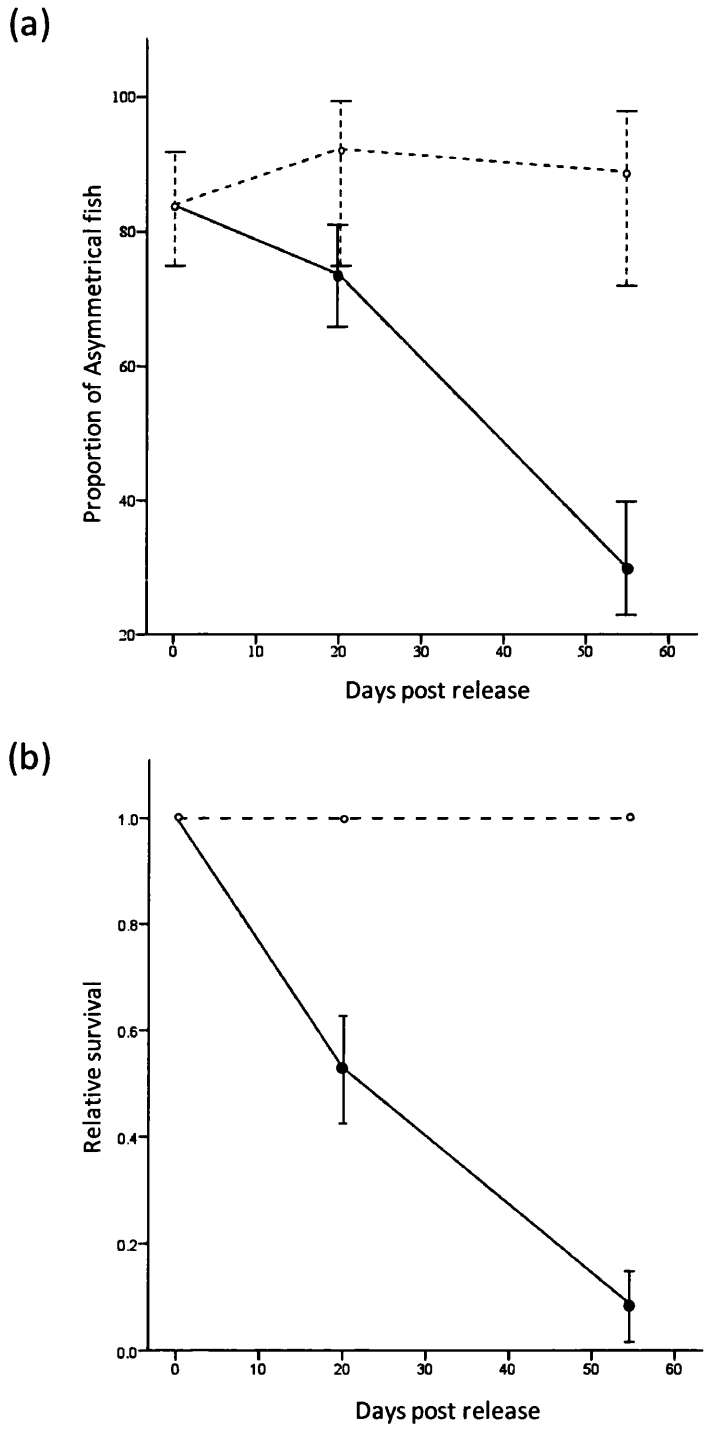


Figure 3.6. Temporal change in (a) proportion of juvenile Atlantic salmon that are asymmetric for at least one meristic trait (± 95 binomial CI) amongst hatchery controls (○) and field recaptures (●), and (b) apparent relative survival (±95 binomial CI) of asymmetrical fish (●) in relation to baseline for symmetrical fish (○).

Chapter IV

Maternal investment and juvenile survival in Atlantic salmon: a field test of the 'Big Old Fat Fecund Female Fish (BOFFF)' hypothesis

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Chapter IV

Maternal investment and juvenile survival in Atlantic salmon: a field test of the 'Big Old Fat Fecund Female Fish (BOFFF)' hypothesis.

ABSTRACT

According to the 'Big Old Fat Fecund Female Fish (BOFFF)' hypothesis, older and larger females may have a fitness advantage because they can produce larger and fitter offspring, which tend to survive better than those of younger mothers, particularly when conditions are harsh. However, this has seldom been tested under natural conditions due to the difficulty of relating embryo size to subsequent performance in the wild. Maternal age and investment in Atlantic salmon (*Salmo salar*) was manipulated by varying the time wild mothers were fed in captivity (from 2 to 26 months), thereby creating mothers of increasing age that produced embryos of increasing size. Resulting juveniles were then released in four natural streams that differed in habitat quality, predator pressure and inter-specific competition, and employed DNA parentage assignment to relate juvenile survival to maternal identity and egg size. There was no evidence of genotype x environment interaction, but found instead was a strong positive effect of egg size on apparent survival that was independent of habitat quality. Thus, juveniles derived from large eggs that had originated from older mothers survived better than those derived from younger, smaller mothers under all conditions, providing support to the BOFFFF hypothesis. The results of this study have implications for fisheries and conservation and suggest that harvesting large females may have a disproportionate negative impact on recruitment and population persistence because larger females are not only more fecund, they also appear to produce larger, and fitter offspring.

INTRODUCTION

For species with limited or no parental care, fitness during early life can depend critically on the quality of maternal egg provisioning, i.e. on the size and energy content of the eggs (Bagenal, 1969; Bagenal and Braum, 1978). Among many fishes, larger females generally produce larger eggs (Berg *et al.*, 2001; Reid and Chaput, 2012) and embryos originating from large eggs often have a survival advantage, especially when environmental quality is poor (Einum and Fleming, 1999; Gregersen *et al.*, 2009; Rollinson and Hutchings, 2010). However, exceptions occur, and for some species the opposite may be true because small larvae benefit from less predation (Litvak and Leggett, 1992), while for other species, a trade-off may exist between attaining a large body size and increasing the length of the growing season (Taylor and Gabriel, 1992; Folkvord *et al.*, 2014). For example, among Atlantic salmon (*Salmo salar*) the benefits of attaining a large body size may be offset by an increased risk of mortality at sea (Cunningham *et al.*, 2013), and the trade-off that exists between egg number and egg size means that large eggs may not always be favoured in all environments (Régnier *et al.*, 2013).

There is supposed to be an optimal egg size that maximizes maternal fitness due to the trade-off that exists between egg size and number (Smith and Fretwell, 1974), but whether there is also an optimal egg size that maximizes offspring fitness is less clear. According to the 'Big Old Fat Fecund Female Fish (BOFFF)' hypothesis (Field *et al.*, 2008; Hixon *et al.*, 2014; Saenz-Agudelo *et al.*, 2014), the contribution of old females to recruitment may be disproportionately high not only because they tend to be larger - and therefore more fecund, but also because they may produce larger, fitter embryos. Yet, several studies suggest that large eggs do not always result in higher fitness (Régnier *et al.*, 2012a) probably because there are multiple optima for egg size, depending on environmental conditions (Einum and Fleming, 1999). Indeed, fish reared in hatcheries often produce small eggs, probably because any survival advantage conferred by a large embryo size disappears in captivity, where selection for fecundity appears to drive the evolution of egg size (Heath *et al.*, 2003).

Intraspecific phenotypic variation is high among many temperate fishes (Dahl *et al.*, 2006) and is thought to have evolved in response to the high levels of spatial and

temporal variation that characterize many aquatic environments, as it enables individuals to cope with changing and unpredictable conditions (Evans *et al.*, 2010). For example, in many temperate rivers, water temperature, water flow, predation intensity or levels of interspecific competition differ markedly across relatively small temporal and spatial scales, and these can act as agents of selection, shaping fish phenotypes and resulting in local adaptations (Garcia de Leaniz *et al.*, 2007a; Garcia de Leaniz *et al.*, 2007b; Fraser *et al.*, 2011). Phenotypic variation within populations can be maintained by phenotypic plasticity, environmental heterogeneity, as well as by genetic and non-genetic (parental) effects (Keeley *et al.*, 2007), and discriminating between these is key for understanding the evolutionary potential of populations (Crespel *et al.*, 2013).

One way to discriminate between the underlying basis of phenotypic variation is through the analysis of genotype–by–environment interactions (G x E) (Evans *et al.*, 2010) and the assessment of reaction norms, i.e. examining how different genotypes give rise to different phenotypes depending on environmental conditions (Crespel *et al.*, 2013; Harney *et al.*, 2013). Genotype–by– environment interactions occur when different genotypes vary in the way they respond to environmental variation, producing different phenotypes under different environments (Khaw *et al.*, 2012). In addition to the effect of current environmental conditions, phenotypic variation can also vary depending on the environment experienced by the parents (Einum and Fleming, 1999; Evans *et al.*, 2014), brought about by non-genetic mechanisms of inheritance (Bonduriansky *et al.*, 2012; Salinas *et al.*, 2013). Such trans-generational, non-genetic mechanisms of inheritance are thought to have arisen in response to large environmental variation, and constitute a likely mechanism for the rapid phenotypic divergence observed among fish reared in captivity (Pulcini *et al.*, 2013). For example, rearing fish under semi-natural conditions increases the survival of their offspring when they are released into the wild (Evans *et al.*, 2014), highlighting the important effects that variation in parental conditions can have on offspring fitness.

Do different environments select for different egg size, i.e. are there genotype–by–environment interactions for optimal embryo size? This has seldom been tested under natural conditions due to the difficulty of relating embryo size to subsequent performance in the wild (Saenz-Agudelo *et al.*, 2014), and never along a gradient of

environmental quality. Here, maternal investment in Atlantic salmon was manipulated by varying the length of time that wild females from the same genetic background were fed in captivity, which resulted in three groups of eggs of increasing size. The offspring were then released into four natural streams, recaptured twice over their first summer, and survivors were genetically assigned to individual mothers of known egg size. The expectation was that juveniles originating from larger, older females would display higher survival and attain a larger size owing to maternal effects linked to egg size. It was also expected that different river environments might select for different phenotypes, resulting from genotype x environment interactions.

METHODS AND MATERIALS

Experimental crosses and manipulation of maternal investment

To manipulate maternal investment, crosses were generated with anadromous Atlantic salmon females that had been fed in captivity for different lengths of time before spawning at the Natural Resources Wales (NRW), Cynrig Fish Culture Unit (Brecon, Wales). Six females that had been caught directly at a fish trap in the River Taff (S. Wales) and had therefore not been fed (mean fork length 69.8 cm ± 56.36 SD) were employed as controls. Additionally, used as impact groups were six females from the same origin that had been kept in the hatchery after they first spawned and subsequently fed for 14 months (mean fork length 68.1 cm ± 66.62 SD), and six females that had been kept in the hatchery and subsequently fed for 26 months (mean fork length 75.9 cm ± 80.54 SD). The females were crossed with twelve unfed wild males (mean fork length 68.3 cm ± 11.0 SD) according to a split-brood design consisting of two sires and three dams (one from each experimental group, Fig. 4.1) on 12th-19th December 2012. Each cross was replicated 6 times, generating a total of 36 families. All the fish used in the crosses were derived from wild parents, had the same genetic background (R. Taff) and were caught at the same place in the River Taff before being crossed at the NRW hatchery.

The eggs were then incubated at the hatchery on a flow-through system at ambient temperature (5.83 °C ± 1.91 SD). To estimate embryo size, a sample of 48 eggs from each female were water hardened and weighed to the nearest 0.001g. Families were kept separated using modified incubation trays until first feeding (30th April 2013) and monitored daily for mortalities so that accurate counts could later be made. After two months, families were combined by maternal group (unfed controls, fed for 14 months, fed for 26 months) and distributed evenly into six 2 m^2 tanks (density ~ 1.77 g L-1) and fed at 2.0-3.5% body weight day^{-1} under natural photoperiod (16:8h light: dark) until late June 2013. On 25th June 2013, 5000 0+ fry of each female group were accurately counted and combined into a single tank (total = 15,000 fry) and this was repeated for each of the four stocked sites (60,000 fry in total). Fish were released along 50 metre sections of four first order stream sites on the headwaters of the river Taff between 27th June and 1st July. Release sites were selected based on the absence of salmon spawning due to impassable barriers and

their location along an altitudinal gradient (from 280 m to 153 m above sea level) to maximize environmental variation and differed in habitat quality, predation risk and intra-specific competition (Fig. 4.2., Table 3.1) as described in Stringwell *et al.* (2014). Experimental fish were sampled using semi-quantitative point electro-fishing along 6 x 50m stretches evenly distributed throughout the stretch of river (starting from 50 m downstream of the stocked area in order to ensure recapture of fry which may have dispersed downstream (Höjesjö *et al.*, 2011)) carried out from bank to bank in a zigzag fashion to cover all micro-habitats. A sample of 100 fish from each site were recaptured approximately 20 days post-release and again at 55 days post-release for parentage assignment.

Parentage assignment

DNA was extracted from adipose fin clips and muscle tissue from all broodstock and recaptured fry using the Nexttec™ 1-Step Tissue & Cells kit (Nexttec Biotechnologie, UK) following manufacturer instructions. Amplifications were carried out using the QIAGEN PCR multiplex kit (Qiagen Ltd, Manchester, UK) following the recommended reaction protocol, with volumes scaled down to a total reaction volume of 8µl. Each reaction included 2µl of purified DNA, 4µl of QIAGEN multiplex mix, 0.8 µl of primer mix and the rest up to 8µl was made up with sterilized distilled water. All the fish were typed for 13 highly polymorphic microsatellites, chosen on the basis of reliability and variability (Ellis *et al.*, 2011) multiplexed in two different polymerase chain reaction (PCR) protocols (Table 4.1). PCR was performed in a 2720 Thermal Cycler (Applied Biosystems) and conducted as follows: 15 min initial activation at 95°C followed by 8 cycles of 94°C for 30 s, 64°C (decreasing 1°C on each cycle) for 90 s and 72°C for 90 s. Then 24 cycles of 94°C for 30 s, 56°C for 90 s and 72°C for 90 s. Final extension was conducted for 10 min at 72°C. Forward primers were fluorescently labelled which enabled the PCR products to be compared according to the 500(-250)LIZ™ size standard using an ABI 3730 automated sequencer. Alleles were automatically called and manually checked in GeneMapper v3.0 (Applied Biosystems). Juveniles which did not amplify at 7 or more microsatellites were removed from the analysis. Parental assignments were performed for each individual fry using the maximum likelihood procedure implemented in CERVUS V3.0 (Kalinowski *et al.*, 2007) using 10 microsatellites,

selected for a high level of variability. Microsatellites were excluded from the analysis in cases where they showed low diversity and evidence of null alleles (Table 4.2). The analysis was run with a simulation of 10,000 offspring, a minimum of 7-typed loci, with the proportion of parents sampled at100% and a typing error of 0.01. The exclusion-based procedure in PAPA V2.0 (Duchesne *et al.*, 2002) was also implemented to verify the crosses from CERVUS. Combinations of 8 - 10 successfully amplified microsatellites were used for the analysis with 350 pseudo-offspring generated at 1000 iterations. Parameters were defined as a uniform error distribution with an error sum of 0.02 distributed over all non-focal alleles. Exclusion-based family assignment simulations in PAPA predicted a ~99% success rate of unambiguous parentage assignment to a single family using the proposed marker combination. Juveniles that did not assign with 95% confidence to a known family in either programme were excluded from all further analysis.

Fry relative survival

Estimates of the number of fry stocked from each family were obtained from egg counts and daily mortalities recorded during the first two months of rearing. After two months, and due to logistic constraints, fry from each of the three maternal origins were combined and it was assumed that mortalities were the same for each family until stocking. This is a reasonable assumption given that subsequent mortalities were low, as is typically the case after the critical period for salmonid survival has passed (Jensen and Collins, 2003; Julien and Bergeron, 2006). Estimates of relative survival I (at family and female levels) were calculated as the ratio between the relative abundance of fish recaptured from each family and the relative abundance of each family at stocking (i.e., I = % contribution to recaptures / % contribution to stocking) under the assumption that a higher relative abundance reflected a higher survival.

Morphometric analysis

At each recapture event a sample of 30 fish from each of the four stocking sites were sampled for morphometric analysis. Fish were photographed (Canon EOS 400D, www.canon.com; 90mm TAMRON SP Di 1:1 macro, www.tamron.eu/uk) from a fixed distance, facing left and with their fins extended, against a standard background

fitted with a scale bar. For each specimen, 19 landmarks used in previous studies (Blanchet *et al.*, 2008; Pulcini *et al.*, 2013) were digitized using the tpsDig 2.16 software (Rohlf, 2004). To correct for possible bias due to body bending, the unbend application of the tpsUtil programme (Rohlf, 2010) was employed, using three additional landmarks along the lateral line to generate corrected landmark coordinates (Haas, 2011). Co-ordinates were then imported into the software MorphoJ for procrustes superimposition (Klingenberg, 2011), which computes an average shape to which specimens are aligned in order to remove the effect of size from the study of morphological variation (Vehanen and Huusko, 2011). Principal component analysis (PCA) was carried out on the covariance matrix followed by mixed linear models on the first two PCA scores to assess the effects of the time spent in the hatchery by the mothers, the time fry spent in the wild and the sites the fry were stocked into on the major features of body shape variation. Measurements of fork length (nearest 0.005 mm) were also calculated from the photos using ImageJ (Abràmoff *et al.*, 2004) as per Stringwell *et al.*, (2014).

Fluctuating asymmetry

Fluctuating asymmetry (FA) was assessed in relation to three bilateral meristic structures fixed in formaldehyde and viewed under an Olympus SZ40 stereo microscope (www.olympus.co.uk) at 4x magnification: (1) number of gill rakers in the upper and lower sections of the first gill arch, (2) number of rays in the pectoral fins, and (3) number of rays in the pelvic fins. Fin ray counts were recorded disregarding any branching, scoring only the base of each ray (Stringwell *et al.*, 2014).

Statistical analysis and model construction

Linear mixed models (lme) were employed with the R. 3.0.0 statistical package 'nlme' to model relative fry survival, body shape and body size in relation to maternal egg weight, maternal rearing time (time spent in captivity, 2, 14 and 26 months), time fry had spent in the wild before recapture (20 or 55 days), and site of release (S1-S4), whilst controlling for the effects of maternal identity as a random factor. Model simplification was carried out by stepwise deletion of non-significant terms beginning with the maximal model using maximum likelihood (ML), and the final,

minimal adequate model was refitted by Restricted Maximum Likelihood (REML) following (Crawley, 2007). To model variation in the proportion of asymmetrical individuals a generalized linear mixed model with random effect (GLMM with binomial error) using the glmer function from the 'lme4' package (Bates *et al.*, 2012) fitted by Laplace approximation was used to assess the effect of female rearing environment, time fry spent in the wild and site of release with maternal identity as a random factor.

In the results section the t- and P-values are presented for all the fixed factors and interactions in the optimal model. Results from the initial models are presented in Appendix I (g-i). All post hoc tests between the female rearing groups were done using the ghlt function from the 'multcomp' package (Hothorn *et al.*, 2008) for multiple comparisons of the mean using Tukey contrasts.

RESULTS

Egg size and fecundity

An overall positive effect of female rearing time was found ($F = 101.28$, $P < 0.001$). Female kelts fed in captivity for 26 months produced larger eggs than control, unfed females captured directly from the wild (Table 4.2), indicating that our experimental manipulation of rearing environment had resulted in differences in maternal investment (see Fig. 1.3a in Chapter 1). An overall positive effect of female body size on the size of eggs was detected as well as a significant interaction between female rearing time and body size (Table 4.2). Pairwise comparisons indicated a significant difference between 2 months and 14 months ($t = -2.52$, $P = 0.032$), 2 months and 26 months ($t = 2.73$, $P = 0.018$) and between 14 months and 26 months ($t = 5.56$, $P < 0.001$).

For the seven females examined in both experimental years, egg weight significantly increased with female fork length (see Table 1.1b and Fig. 1.4 in Chapter 1). There was also a significant effect of the year the experiment was carried out on egg size and a significant interaction between year and female fork length with the effect of female body size being greater in year 2 to that in year 1 (see Table 1.1b in Chapter 1). Control females and kelts did not differ significantly in relative fecundity ($t_{7.5} = -0.056$, $P = 0.957$; kelts = 1,477 ± 76 eggs/kg; controls = 1,487 ± 154 eggs/kg).

Parentage assignment

Allele frequency analysis in CERVUS confirmed that allele frequencies did not deviate significantly from Hardy-Weinberg equilibrium for most loci (Table 4.3). Out of a total of 770 recaptured fry, 43 did not amplify at 7 or more microsatellites and were removed from the analysis. Of the 727 genotyped fry a total of 671 were successfully assigned to true parental crosses (92.3% successful allocations) at 95% confidence level (Appendix II). PAPA assigned 95.4% of fry to the same parental cross as CERVUS.

Relative fry survival

The progeny of three females was not represented among fry recaptures in any of the four sites sampled (two females were absent during the first recapture, 20 days post-stocking, and one female was absent during the second recapture, 55 days post-stocking; Fig. 4.3). Linear mixed effects modelling indicated that fry survival depended strongly on maternal identity, which explained 42.4% of variation, and that egg weight was the only significant predictor of fry survival (Table 4.4, Fig. 4.4). Survival was not affected by site of release (Appendix I-g), indicating that there were no genotype x environment interactions in fry survival. In general, families which did well in one habitat did well in most habitats and *vice versa* (Fig. 4.3).

Maternal effects on body shape and body size

Results from linear mixed model on PC1 scores indicated that body shape of recaptured fry differed significantly depending on the time elapsing since stocking (Table 4.5a), which affected body shape differently depending on site of release (Table 4.5a and Fig. 4.5). No effect of maternal group was detected and so was removed from the model (Appendix I-h). For PC2, there was a significant effect of site of release (Table 4.5b) and a significant interaction between site and time elapsing since release (Table 4.5b and Fig. 4.5) and no maternal effect was detected (Appendix I-h).

Variation in body size of recaptured fry was found to depend on maternal group, time elapsing since release, egg weight and the interaction between site and time elapsing since release (Table 4.6). An overall significant effect of female rearing time on offspring body size was found ($F_{2,195} = 5.26$, $P = 0.006$). Pairwise comparisons indicated a significant difference between 2 months and 14 months ($z = 2.92$, $P = 0.010$) and 2 months and 26 months ($z = 2.38$, $P = 0.046$) but no difference between 14 months and 26 months ($z = -0.65$, $P = 0.795$).

Fluctuating asymmetry

Results from generalized linear models with random intercept indicate that asymmetry of fry decreases with time spent in the wild ($z_{285} = -6.563$, $P < 0.001$), but

no effects of maternal origin, site of release, or any interactions were found (Appendix I-i).

DISCUSSION

By making use of DNA parentage assignment it was possible to compare the performance of juvenile Atlantic salmon derived from different mothers under different habitats, and therefore test what effects variation in maternal provisioning (i.e. egg size) may have had on offspring fitness in different environments. This is one of the first times that genotype x environment interactions for maternal effects on fish fitness have been tested under a range of natural stream conditions. Performance in the wild of brown trout (*Salmo trutta*) has been examined in relation to paternal migratory life history (Höjesjö *et al.*, 2011). Although, no paternal effect was found a significant effect of the female parent suggested maternal and/or genetic effects.

Previously, the challenge of linking juvenile fitness with variation in maternal provisioning had proved largely intractable in the field, as eggs are typically too small to be marked and juveniles could not be traced to their mothers (Skalski *et al.*, 2009); inferences had to be drawn from laboratory (e.g. Régnier *et al.*, 2010; Houde *et al.*, 2011; Régnier *et al.*, 2012a; Régnier *et al.*, 2013) or - at best - semi-natural conditions (Einum and Fleming, 1999; Einum, 2003; Régnier *et al.*, 2012b). It is only recently that DNA parentage assignment has made it possible to relate variation in maternal investment to offspring fitness under natural conditions (Serbezov *et al.*, 2010; Beldade *et al.*, 2012; Saenz-Agudelo *et al.*, 2014). Using such an approach, the results of this study indicate that relative survival of hatchery-reared juvenile Atlantic salmon during the first growing season - a critical period for survival in salmonids and many other fishes (Elliott, 1989; García de Leániz *et al.*, 2000; Nislow *et al.*, 2004), depends on maternal provisioning, as seen in other fish species (e.g. Berkeley *et al.*, 2004; Taborsky, 2006). Thus, differences in the ratio of the number of stocked fish to subsequent recapture (our proxy for apparent survival) ranged from 0 to 3.2 among dams, indicating that some females contributed no offspring, while others produced fish that survived more than three times better than would be expected by chance alone. As with many other studies in natural streams, it was not possible to tease apart emigration from mortality, but the assumption that more recaptures represent greater survival is a reasonable one, as shown recently in the same study system (Roberts *et al.*, 2014). In the study conducted by Roberts *et al.*, (2014), Atlantic salmon fry were reared in environmentally enriched or standard hatchery tanks before release into the wild. After 97 days spent in the wild, there was no

evidence of differential migration between the two groups within the 3 km of the study reach. Dispersal of salmonid fry has however been found in released fish, with an average downstream movement of 40 m (Höjesjö *et al.*, 2011). Electrofishing of the sites in this study began 50 m further downstream of the release point in order to account for fry dispersal.

The manipulation of maternal rearing environment in this study indicates that feeding female salmon to satiation in captivity after their first spawning (i.e. kelt reconditioning) resulted in larger eggs, which resulted in larger alevins, which in turn grew and survived better in the wild, thereby providing some support to the BOFFF hypothesis (Field *et al.*, 2008; Hixon *et al.*, 2014; Saenz-Agudelo *et al.*, 2014). It is also apparent, at least for the seven females that could be studied over two consecutive years, that an extra year of growth in captivity translated into larger eggs but without significantly increasing fecundity, thereby providing additional support for the idea that mothers may adjust egg provisioning, not only according to size, but also depending on age (Berkeley *et al.*, 2004; Palumbi, 2004). A positive effect of egg weight on relative survival (Einum and Fleming, 2000b; Johnston and Leggett, 2002; Krist, 2011) is likely to have resulted from an increase in maternal investment (Skaala *et al.*, 2012), most likely mediated by maternal age, body size, and diet (Koops *et al.*, 2003). Although egg composition was not measured, egg size has been found to be a good predictor of energy content in salmonids (Régnier *et al.*, 2012b; Mitchell *et al.*, 2014) and kelts fed in captivity in this study produced larger eggs than control (i.e. unfed) maiden females, after statistically controlling for maternal identity and variation in female body size. Although eggs were not directly stocked into the wild and fry were not released until two months after first feeding, the effect of maternal provisioning was still evident at the time of stocking as fry from large eggs were larger in body size. Similar findings have been observed for nine-spined stickleback (*Pungitius pungitius* Linnaeus, 1758), whereby offspring body size from 'giant' mothers appeared to be mediated through differences in egg size (Ghani *et al.*, 2012). As offspring fitness has been found to depend mainly on egg size in a diversity of organisms (Fox and Mousseau, 1996; Einum and Fleming, 2000a), it is reasonable to believe that the association between egg size and survival found in this study is representative of what would have been observed if offspring had been stocked as eggs rather than fry.

Models of optimal egg size predict that females may maximize fitness by producing more and smaller eggs when under favourable conditions, and fewer and larger eggs under harsher conditions (Smith and Fretwell, 1974). For some species this appears to hold true. For example, egg size increases survival in beetles when conditions are poor, but not when conditions are good (Fox and Mousseau, 1996), and the same appears to be the case in some salmonid studies (Einum, 2003). However, the results of the present kelt reconditioning experiment appear to contradict this tenet, as under favourable growing hatchery conditions, most kelts produced larger, not smaller, eggs while dam-specific relative survival was the same across the four study sites. It is possible that females adjust maternal investment depending on environmental conditions likely to be experienced by their offspring (Einum and Fleming, 2002; Marshall and Keough, 2008; Rollinson and Hutchings, 2010), using growth cues provided during maturation, but perhaps also during their own juvenile phase (Taborsky, 2006). For example, female Atlantic salmon which grow slowly when young tend to produce larger eggs and fitter offspring which survive better (Burton *et al.*, 2013b) though this is unlikely to have played a role in this study.

It was found in this study that variation in maternal investment (through variation in egg weight) was the sole determinant of survival in the wild, which was independent of habitat quality, as evidenced by the lack of statistical significance for location of release, or their interactions. Such absence of a genotype x environment interaction in survival is perhaps surprising, considering that different stocking sites differed substantially in habitat quality, predation pressure and degree of intra-specific competition, and interacted with time since release to produce fish of different body shapes (Stringwell *et al.*, 2014). Body shape is believed to be under strong selection as it greatly affects swimming performance, but the relation between body shape and fitness is likely to be a complex one. Thus, while a streamlined shape may aid swimming performance, a deeper body and larger fins may confer superior burst swimming speed and a competitive advantage against competitors (Swain *et al.*, 1991). Variation in fry body size had a strong maternal component and was affected by release site but no G x E interaction was detected, suggesting that in this case both maternal and environmental effects are present but they may have contributed additively to body size over time (Salinas *et al.*, 2013).

The development of bilateral structures on opposite sides of an organism is controlled by the same genes, and any deviations from bilateral symmetry are thought to result from environmental and genetic stressors (Johnson *et al.*, 2004). A positive effect of time spent in the wild on the proportion of symmetric individuals was found, as reported previously (Stringwell *et al.*, 2014), but no maternal effect was detected. The absence of maternal effect on fluctuating asymmetry, or the lack of clear relationship between body shape and fry survival would seem to merit further study.

Taken together, the results demonstrate the existence of transgenerational effects on offspring fitness in juvenile Atlantic salmon, seemingly mediated by adjustments in egg size. Similar transgenerational effects on offspring fitness have been noted recently on this species, whereby the environment experienced by mothers had a marked influence on offspring survival, presumably mediated via phenotypic changes in morphology and behaviour (Evans *et al.*, 2014). In this study, however, the only direct effect of maternal rearing environment can be accounted by variation in egg size. The test of the BOFFF hypothesis suggests that egg size is positively correlated to fitness, and to female age and body size. Such findings substantiate concerns about the implications of targeting large female spawners in exploited salmonid populations (Consuegra *et al.*, 2005; Saura *et al.*, 2010), because harvesting such fish will likely have a disproportionate impact on recruitment, not only via lost fecundity, but as this study suggests, also because this removes the largest, fittest embryos from the population.

ACKNOWLEDGEMENTS

I am grateful to a number of Swansea University volunteers and to staff at Natural Resources Wales for rearing the fish at Cynrig and for help with the sampling in the River Taff. This work was part funded by the European Social Fund (ESF) through the European Union's Convergence programme administered by the Welsh Government.

Table 4.1. Characteristics and references of the 13 different microsatellites used in two multiplexes. Primer name, repeat motif (RM) and size range.

Primer	RM	Size	Reference
Multiplex I			
SSsp2210	4	104-164	Paterson *et al.* (2004). Mol.Ecol.Notes 4:160-162
Ssa202	4	200-320	O'Reilly *et al.* 1996. CJFAS 53:2292-2298
SSspG7	4	112-214	Paterson *et al.* (2004) Mol.Ecol. Notes 4:160-162
Sp2201	4	259-371	Paterson *et al.* (2004) Mol.Ecol. Notes 4:160-162
SsaD144	4	104-302	King *et al.* (2005) Mol. Ecol. Notes 5, 130
Sasa-UBA	2	110-146	Grimholt *et al.* (2002). Immunogenetics 54: 570-581
Sp1605	4	222-254	Paterson *et al.* (2004). Mol.Ecol.Notes4:160-162
Multiplex II			
Sp2216	4	202-305	Paterson *et al.* (2004). Mol.Ecol.Notes 4:160-162
Ssa197*	4	135-279	O'Reilly *et al.* (1996). CJFAS 53:2292-2298
SSsp3016	4	70-114	Gilbey *et al.* (2004). Animal Genetics 35: 98-105
Sasa-DAA	10	208-368	Grimholt *et al.* (2002). Immunogenetics 54: 570-581
Ssa289*	2	110-132	McConnell *et al* (1995). CJFAS 52: 1863-1872.
Ssa171	4	193-272	O'Reilly *et al* (1996). CJFAS 53:2292-2298

Table 4.2. Results of linear model examining the influence of body size and time Atlantic salmon females (n = 18) spent in hatchery environment on egg weight (n = 864); Estimates of the magnitude of the effect of each parameter on egg weight (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced. Pairwise comparisons indicated a significant difference between 2 months and 14 months (t = -2.52, P = 0.032), 2 months and 26 months (t = 2.73, P = 0.018) and between 14 months and 26 months (t = 5.56, P < 0.001).

Parameter	**β**	**βSE**	**t**	**p**
Intercept	-2.429	0.318	-7.637	**< 0.001**
14 Months	-1.125	0.447	-2.519	**0.012**
26 Months	1.109	0.406	2.734	**0.006**
Body size (FL)	0.524	0.112	4.685	**< 0.001**
14 months x Body size	0.397	0.157	2.523	**0.012**
26 months x body size	-0.370	0.142	-2.609	**0.009**

Table 4.3. Allele frequency analysis results from CERVUS. K = Number of alleles at the locus; N = Number of individuals typed at the locus; H_o = Observed heterozygosity; H_e = Expected heterozygosity; PIC = Polymorphic information content; F(Null) = Frequency of null alleles.

Locus	K	N	H_o	H_e	PIC	F(Null)
20 d post stocking						
SSsp2210	13	335	0.839	0.821	0.803	-0.0139
Ssa202	14	406	0.823	0.873	0.859	0.0293
SSspG7	23	421	0.888	0.884	0.874	-0.0029
Sp2201	24	410	0.959	0.929	0.923	-0.0170
SsaD144	28	402	0.920	0.923	0.917	0.0007
Sasa-UBA	28	423	0.827	0.845	0.830	0.0087
Sp1605	24	390	0.849	0.806	0.778	-0.0310
Sp2216	16	421	0.895	0.891	0.881	-0.0012
Ssa197	23	423	0.927	0.895	0.885	-0.0191
SSsp3016	26	422	0.922	0.896	0.885	-0.0161
Sasa-DAA	10	417	0.772	0.797	0.769	0.0186
Ssa171	16	417	0.885	0.889	0.877	0.0010
55 d post stocking						
SSsp2210	13	215	0.777	0.796	0.777	0.0039
Ssa202	17	345	0.852	0.876	0.863	0.0124
SSspG7	23	354	0.895	0.892	0.882	0.0022
Sp2201	30	340	0.900	0.932	0.926	0.0167
SsaD144	27	350	0.914	0.928	0.922	0.0066
Sasa-UBA	26	355	0.803	0.854	0.840	0.0289
Sp1605	23	343	0.828	0.798	0.768	0.0240
Sp2216	18	358	0.897	0.885	0.874	0.0069
Ssa197	20	359	0.905	0.898	0.888	0.0058
SSsp3016	26	361	0.939	0.876	0.863	0.0390
Sasa-DAA	13	357	0.768	0.777	0.747	0.0051
Ssa171	16	340	0.847	0.885	0.873	0.0214

Table 4.4. Results of linear mixed-effects model examining the influences on juvenile Atlantic salmon survival released as 0+ fry (n = 144) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on survival (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**B**	**βSE**	**DF**	**T**	**p**
Intercept	-0.783	0.770	126	-1.017	0.311
Egg Weight	14.264	6.249	16	2.283	**0.037**

Table 4.5. Results of linear mixed-effects model examining the influences on body shape of juvenile Atlantic salmon (n = 206) originating from females which had spent varying lengths of time in the hatchery (n = 16). Estimates of the magnitude of the effect of each parameter on body shape (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
a) PC1					
Intercept	0.005	0.002	183	2.908	**0.004**
Site 2	-0.008	0.002	183	-3.481	**< 0.001**
Site 3	-0.013	0.002	183	-5.589	**< 0.001**
Site 4	0.0009	0.002	183	0.364	0.716
Time (DPR)	0.006	0.002	183	3.425	**0.001**
Site 2 x Time (DPR)	-0.007	0.002	183	-3.020	**0.003**
Site 3 x Time (DPR)	-0.005	0.002	183	-2.032	**0.044**
Site 4 x Time (DPR)	0.005	0.002	183	2.100	**0.037**
b) PC2					
Intercept	0.004	0.002	183	2.271	**0.024**
Site 2	-0.0003	0.002	183	-0.144	0.885
Site 3	-0.008	0.002	183	-4.188	**< 0.001**
Site 4	-0.004	0.002	183	-1.889	0.060
Time (DPR)	-0.002	0.001	183	-1.524	0.129
Site 2 x Time (DPR)	0.005	0.002	183	2.189	**0.030**
Site 3 x Time (DPR)	0.005	0.002	183	2.509	**0.013**
Site 4 x Time (DPR)	-0.003	0.002	183	-1.203	0.231

Table 4.6. Results of linear mixed-effects model examining the influences on body size (fork length) of juvenile Atlantic salmon (n = 220) originating from females which had spent varying lengths of time in the hatchery (n = 16). Estimates of the magnitude of the effect of each parameter on body size (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced. Pairwise comparisons indicated a significant difference between 2 months and 14 months (z = 2.92, P = 0.010) and 2 months and 26 months (z = 2.38, P = 0.046) but no difference between 14 months and 26 months (z = -0.65, P = 0.795).

Parameter	**β**	**βSE**	**DF**	**T**	**p**
Intercept	1.653	0.009	196	179.245	**< 0.001**
14 months	0.028	0.010	13	2.924	**0.012**
26 months	0.023	0.010	196	2.377	**0.018**
Time (DPR)	0.066	0.006	196	11.662	**< 0.001**
Site 2	0.010	0.008	196	1.218	0.225
Site 3	-0.0001	0.008	196	-0.019	0.985
Site 4	0.010	0.008	196	1.201	0.231
Egg weight	0.010	0.004	13	2.542	**0.025**
Site 2 x Time (DPR)	-0.040	0.008	196	-4.964	**< 0.001**
Site 3 x Time (DPR)	-0.050	0.008	196	-6.360	**< 0.001**
Site 4 x Time (DPR)	-0.025	0.008	196	-3.033	**0.003**

Figure 4.1.

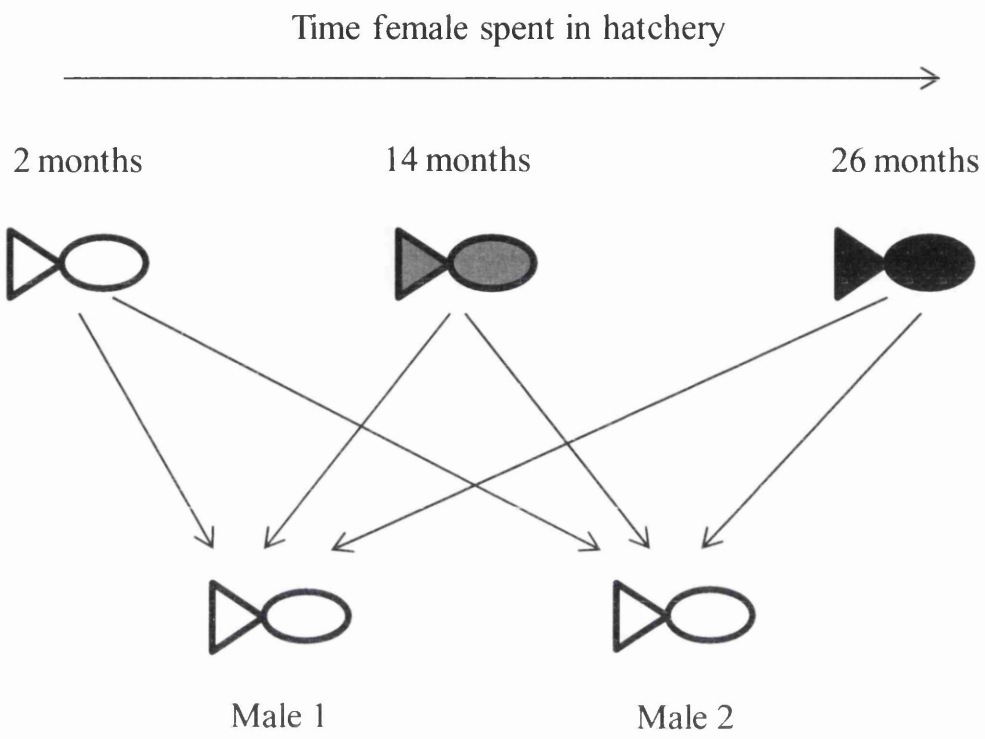

Figure 4.1. Schematic diagram of 3 x 2 split-brood breeding design. Two male Atlantic salmon were crossed with three females (one from each experimental group; 2 months in hatchery (○), 14 months in hatchery (◉) and 26 months in hatchery (●) to produce 6 families.

Figure 4.2.

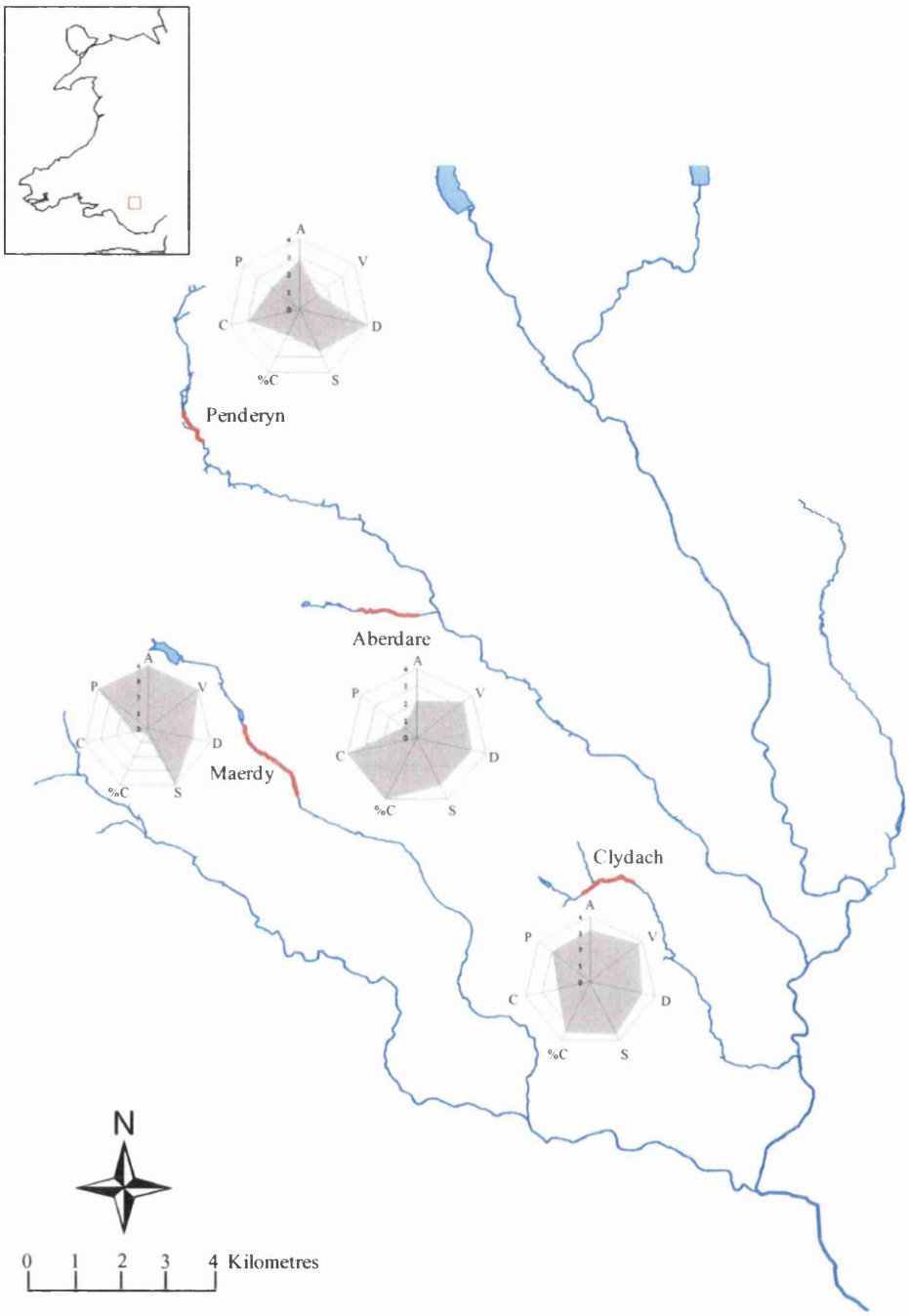

Figure 4.2. Map showing the distribution of the four experimental tributaries of the River Taff, South Wales used for stocking Atlantic salmon 0+ fry in June 2013. Included are the corresponding sun-ray plots illustrating environmental profiles of each site based on seven variables standardised from 0 to 4; A = Altitude (m), V = Velocity (m s^{-1}), D = Depth (cm), S = Substrate size (cm), % C = Cover, C = Competition, P = Predation.

Figure 4.3.

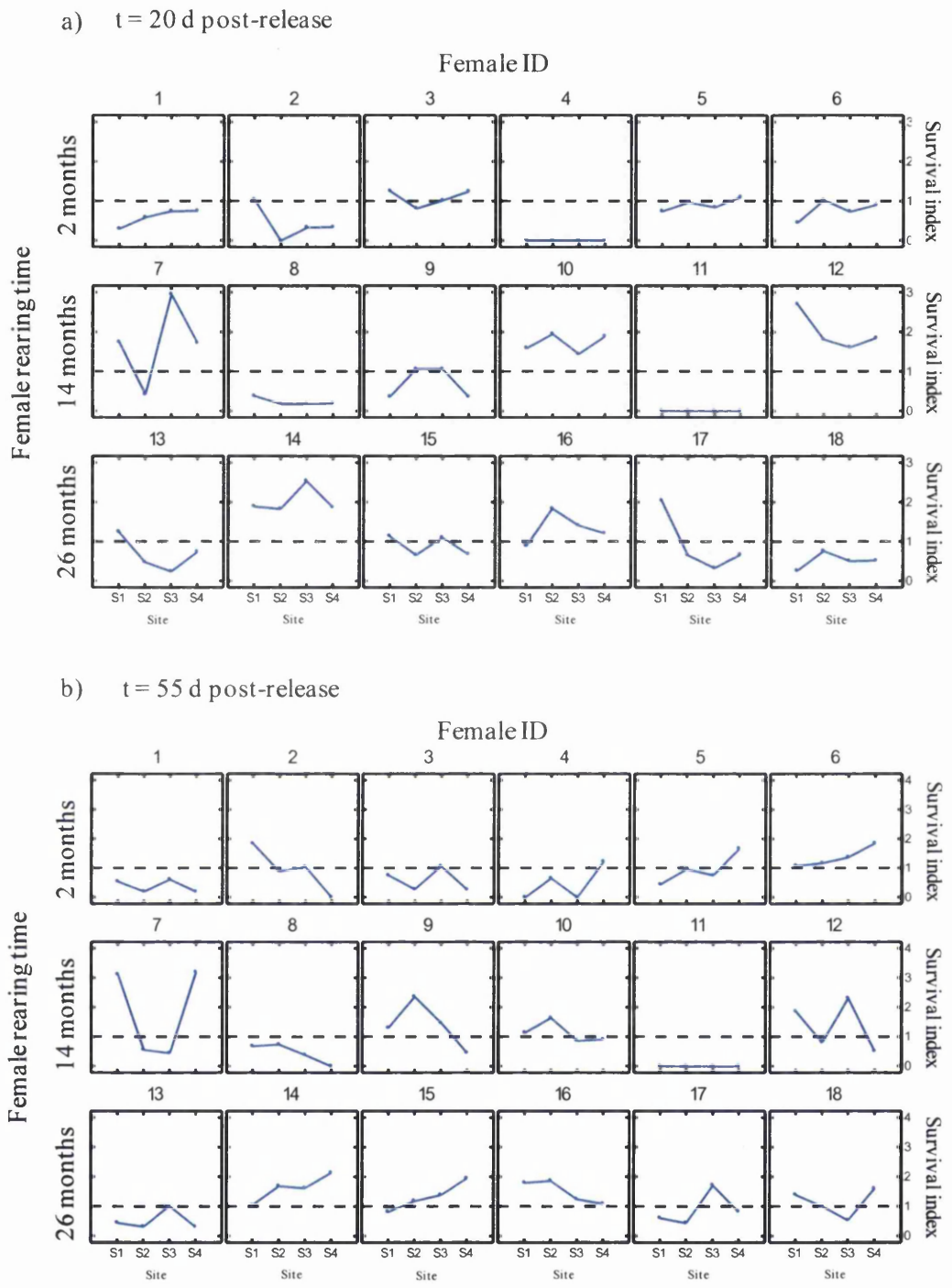

Figure 4.3. Survival index of hatchery-reared Atlantic salmon 0+_ fry which were stocked into four tributaries of the River Taff, South Wales in June 2013 and subsequently recaptured after a) 20 days and b) 55 days in the wild. Fry originated from female Atlantic salmon which spent 2 months (top row), 14 months (middle row) or 26 months (bottom row) in the hatchery environment.

Figure 4.4.

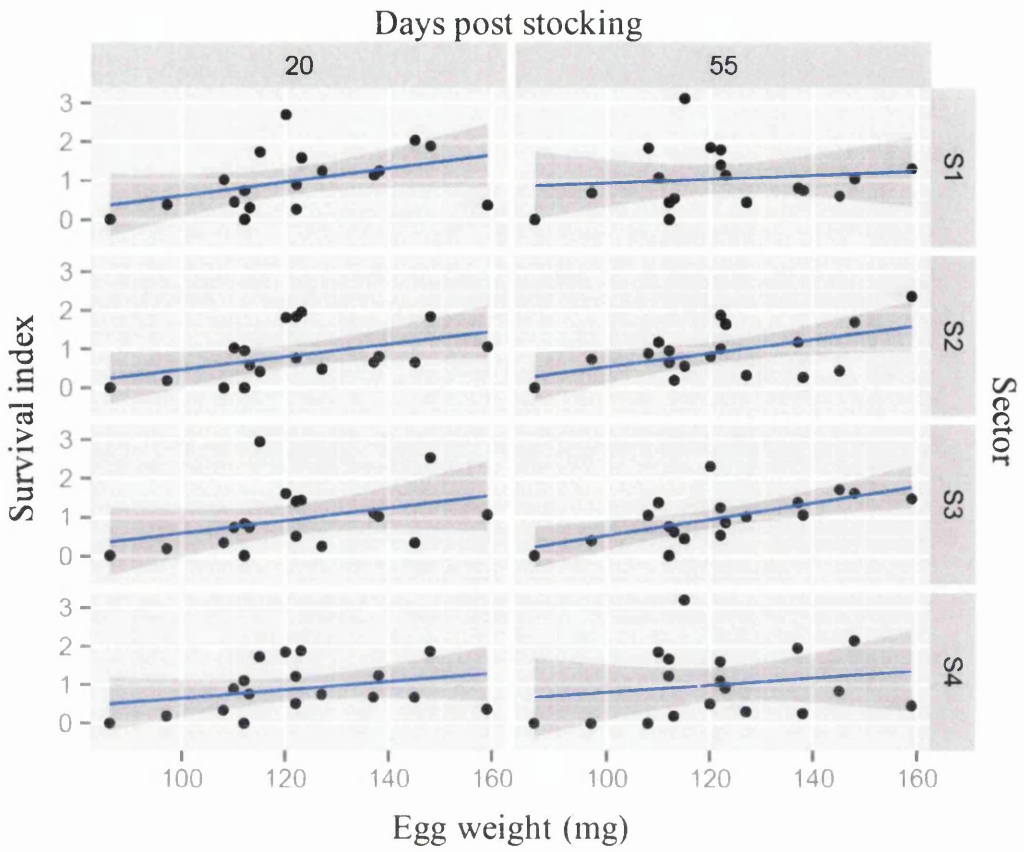

Figure 4.4. Relationship between egg weight (mg) and survival index of 0+ Atlantic salmon fry that were stocked into four tributaries of the River Taff, South Wales in June 2013 and subsequently recaptured after a) 20 days and b) 55 days in the wild.

Figure 4.5.

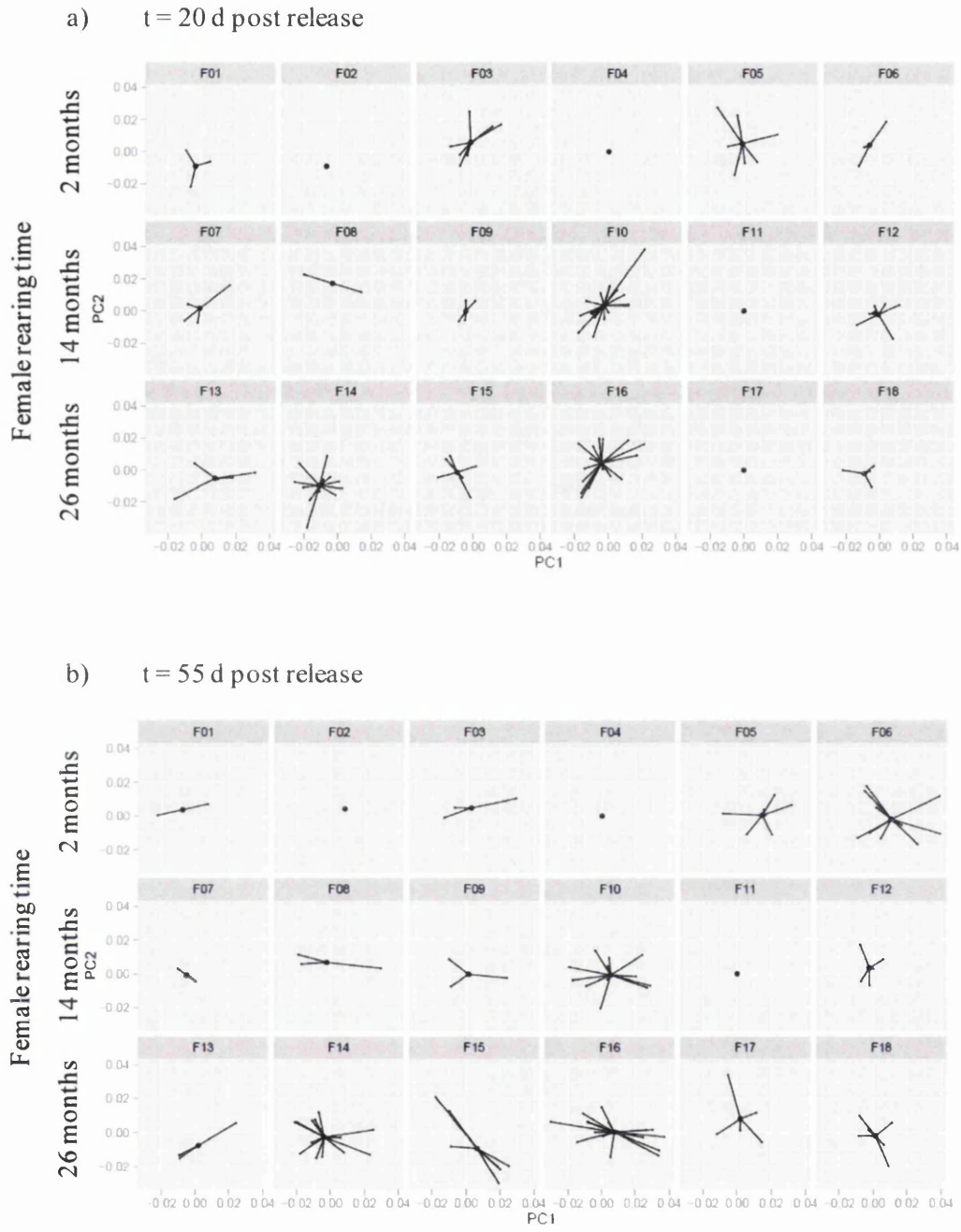

Figure 4.5. Phenotypic variation in morphology of surviving 0+ Atlantic salmon fry that were stocked into tributaries of the River Taff, South Wales in June 2013 and subsequently recaptured after a) 20 days and b) 55 days in the wild. Fry originated from female Atlantic salmon which spent 2 months (top row), 14 months (middle row) or 26 months (bottom row) in the hatchery environment. Depicted are the first two principal components of each recaptured juvenile examined for morphometric variation joined at the mean for each female.

GENERAL CONCLUSIONS

As natural populations decline, captive-rearing programmes have become important components of conservation and management efforts (Williams and Hoffman, 2009). However, the effects of supplementing wild populations with captive-bred organisms are not yet clear. Any negative effects of captive breeding are especially relevant for salmonids due to the huge scale of hatchery programmes employed to compensate for the worldwide decline in the species (Araki *et al.*, 2009). There is very little evidence that the introduction of hatchery-reared salmon has boosted the long-term productivity of wild populations (Fraser, 2008). Yet there is evidence to suggest that supplementation has led to increased risk of disease introductions, increased competition for resources, and genetic changes in the supplemented populations (Evans *et al.*, 2014). The genetic risk results from artificial environments selecting for captive-bred individuals that are maladapted to the natural environment (Stringwell *et al.*, 2014). Therefore, captive-bred organisms could potentially drag down the fitness of the wild populations they are meant to support, even while temporarily boosting their numbers (Araki *et al.*, 2009).

While adaptation may occur via the inheritance of genetically based traits selected during exposure to the hatchery environment, the potential for non-genetic inheritance of traits arising from plastic responses of mothers to a change in environmental variation has also been suggested as a likely mechanism of rapid divergence of captive-reared and wild salmon (Araki *et al.*, 2009). The current thesis aimed to examine the transgenerational effects of rearing wild Atlantic salmon females in a hatchery environment for increasing amounts of time on offspring fitness and survival in the wild. The results of each chapter are summarised below:

1. Egg size is one of the most extensively studied aspects of maternal effects due to the positive correlation that often exists between embryo size and survival (Einum and Fleming, 2000a). Individuals which hatch from small eggs can, however, compensate for the lack of maternal provisioning by increasing their growth rate and gaining prior access to profitable feeding territories (Heath *et al.*, 1999). In this study, females which spent increasing lengths of time in the hatchery produced larger eggs for their body size than those which had spent only a few months in captivity. Egg weight increased with female body size in maiden fish, a relationship often seen

in wild Atlantic salmon (Rollinson and Hutchings, 2011), whereas this relationship weakened for kelts.

In the wild, there is a trade-off in the timing of hatching, as those embryos which hatch early and consume provisions quickly will likely face an increase in predation risk, but may increase their chances of acquiring a profitable feeding territory (Einum and Fleming, 2000b; Garcia de Leaniz *et al.*, 2000). In the present study, large eggs from maiden mothers typically hatched later than those from smaller eggs, however, this relationship was altered when rearing time of mothers was increased. Additionally, utilization of the yolk sac was faster in offspring from mothers which had spent longer in the hatchery, suggesting an increased metabolic rate, a possible consequence of an underlying effect of domestication in the hatchery environment. This could have underlying consequences for fish once released into the wild as individuals with a higher metabolic rate are more likely to have an increased growth rate which can have a direct effect on key life history events such as timing of migration and maturation (Burton *et al.*, 2011). By using individual level measurements of timing of hatch and yolk sac utilization, the present study has shown that the rearing environment of mothers can alter the timing of important life stages and should therefore be considered in the study of egg size evolution.

2. Stress coping styles have been documented in a number of animal species including Atlantic salmon. Correlated behavioural and physiological traits often cluster into two distinct styles, with animals characterised as either proactive or reactive (Koolhaas *et al.*, 1999). In wild salmonids, early emergence from the spawning redd has been associated with a high metabolic rate and increased aggression (Cutts *et al.*, 1998). Maternal effects can also influence the timing of this crucial niche shift as well as metabolic rate through the nutritional provisioning to eggs. By rearing wild females in captivity for varying lengths of time it was possible to manipulate the amount of resources provided to eggs. The results indicate that there is no association between metabolic rate and aggression, which suggests that there may be an uncoupling of strongly associated behavioural and physiological traits of the proactive stress coping style (Vaz-Serrano *et al.*, 2011).

Aggression was inversely related to the length of time mothers spent in a hatchery environment, suggesting that maternal effects may also influence the

correlation between metabolic rate and behaviour. It may be that when provisioning is high, competition for feeding territories may only yield a small gain in fitness for the individual (Drake *et al.*, 2008). The uncoupling of metabolic rate with aggression and the timing of emergence seen in this study might therefore be a consequence of domestication, due to relaxed selection pressures against maladaptive phenotypes in the hatchery environment (Stringwell *et al.*, 2014), giving rise to increasing behavioural variability (McPhee, 2004). The results in this study demonstrate that some behavioural components of stress coping styles can be modified by maternal effects, whereas behavioural plasticity is likely limited (Ruiz-Gomez *et al.*, 2008).

3. Hatchery-reared fish typically perform poorly in the wild, suggesting a phenotypic mismatch to the natural environment. Experiments employing a BACI approach can help to gain a better understanding of the responses of fish to changes in rearing environment. In this study, the phenotypes of salmon fry changed substantially over time in both the hatchery and natural environments and perhaps most interestingly fish in the wild diverged significantly from hatchery fish as early as 20 days post-release. Juvenile salmon became more streamlined, more cryptic, more symmetrical and their caudal fins and opercula regenerated in the wild. The fitness implications of these phenotypic changes are difficult to predict in the wild but are likely to be adaptive because morphology affects swimming efficiency, feeding ability and predator avoidance (Pakkasmaa and Piironen, 2001; Adams *et al.*, 2003; Drinan *et al.*, 2012). It was evident in this study that confinement in hatchery tanks with low water velocity and plentiful food increased fat deposition and resulted in the deepening of the body (Pulcini *et al.*, 2013). Fry released into the wild also displayed darker parr marks than hatchery controls, making them more cryptic and less conspicuous to predators (Culling *et al.*, 2013). This study found significant differences in parr mark contrast between those released into the wild and those retained in the hatchery within just 20 days.

The extent to which these changes were the result of phenotypic plasticity or non-random mortality of maladapted phenotypes is unclear. However, given that there was no mortality in the hatchery, two different mechanisms must have been at work: phenotypic plasticity in the hatchery, and plasticity plus selection in the wild. The development of bilateral structures on opposite sides of an organism is controlled by the same genes, and any deviations from perfect bilateral symmetry are

thought to result from environmental and genetic stressors (Johnson *et al.*, 2004). The meristic structures considered in our study are not plastic but become fixed during early development (Swain and Foote, 1999) and so the observed marked decrease in the frequency of asymmetric individuals in the wild must have been due to a higher mortality of asymmetrical fish relative to symmetrical ones. Evidence for non-random mortality of maladapted hatchery phenotypes was found in our study suggesting that hatcheries generate fish which are phenotypically mismatched to the natural environment. The results indicate that the longer fish remain in the hatchery environment the more dissimilar they become to fish in the wild.

4. Fitness during early life can depend critically on the quality of maternal egg provisioning, i.e. on the size and energy content of the eggs (Bagenal, 1969a; Bagenal and Braum, 1978). Among many fishes, larger females generally produce larger eggs (Berg *et al.*, 2001; Reid and Chaput, 2012) and embryos originating from large eggs often have a survival advantage, especially when environmental quality is poor (Einum and Fleming, 1999; Gregersen *et al.*, 2009; Rollinson and Hutchings, 2011). Among Atlantic salmon the trade-off that exists between egg number and egg size means that large eggs may not always be favoured in all environments (Régnier *et al.*, 2012a). According to the 'Big Old Fat Fecund Female Fish (BOFFF)' hypothesis, the contribution of old females to recruitment may be disproportionately high as they tend to be larger and therefore more fecund, producing larger and fitter embryos (Field *et al.*, 2008; Saenz-Agudelo *et al.*, 2014).

In the present study, there was evidence of a strong positive effect of egg weight on the relative survival of fry, independent of habitat quality and this is likely due to the increased maternal investment (Skaala *et al.*, 2012). Juveniles derived from large eggs that had originated from older mothers survived better than those derived from younger, smaller mothers under all conditions, providing support to the BOFFF hypothesis. A transgenerational effect on body size of fry after time spent in the wild was found in this study where females retained in the hatchery for up to two years produced larger fry than females which had only spent a couple of months in captivity. This may have provided fry from older kelts a competitive advantage over fry from younger mothers. These results suggest that harvesting large females may have a disproportionate negative impact on recruitment through the removal of the largest, fittest offspring from the population.

Overall, the results from this thesis could have important implications. Given that increased maternal exposure to the hatchery environment resulted in larger eggs which ultimately influenced early development and changes in behaviour whilst also increasing survival in the wild, supplementation programmes could have a cumulative impact on wild populations. The consequences for this could be severe as earlier development of offspring can have an effect on later life-history thresholds such as the timing of migration and maturation in salmonids (Wilke *et al.*, 2014). Atlantic salmon typically emigrate from freshwater in the spring after having reached a growth-dependant size threshold (Otero *et al.*, 2014). As rearing mothers in the hatchery for longer is increasing the metabolic and developmental rate of offspring the resulting increased growth rate could have the potential to shift the timing of migration once offspring are released into the wild. Further to this, a study by Araki *et al.*, (2009) found a carry-over effect of captive-breeding over a number of generations which had a negative influence on the size of the wild population in the generation after supplementation. This therefore suggests that the maternal effect imposed on the offspring in this study could also be passed on to the subsequent generation.

This thesis also found a phenotypic mismatch of hatchery-reared salmon fry to the wild environment but also found that after a period of time the phenotypes of fish changed to suit the environment that they were in. A number of studies have found that environmental enrichment improves a number of phenotypic traits known to be important for survival in the wild such as, foraging efficiency (Brown, 2003), exploratory behaviour (Berejikian *et al.*, 2003; Braithwaite and Salvanes, 2005) and risk taking behaviour (Roberts *et al.*, 2011). This study therefore adds to the increasing evidence for the benefits of enrichment in reverting human altered phenotypes under relaxed selection scenarios (Lahti *et al.*, 2009) and highlights the need to consider multiple phenotypic traits in supplementary programs. Minimizing maternal exposure to captivity and maximising juvenile exposure to semi-natural rearing should therefore be an important component in captive rearing programs for the supplementation of endangered populations.

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APPENDIX

Appendix I

a) Results of initial linear mixed-effects model examining the influences on the timing of hatching of Atlantic salmon alevins (n = 1096) originating from females which had spent varying lengths of time in the hatchery (n = 24). Estimates of the magnitude of the effect of each parameter on timing of hatching (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	2.705	0.030	1068	89.026	**< 0.001**
14 months	-0.001	0.003	20	-0.200	0.844
26 months	-0.008	0.004	20	-2.022	0.057
Egg size category	0.0005	0.001	1068	0.686	0.493
Egg Weight	-0.005	0.001	1068	-3.387	**0.001**
14 months x Egg Weight	0.003	0.002	1068	2.025	**0.043**
26 months x Egg Weight	0.006	0.002	1068	2.983	**0.003**

b) Results of initial linear mixed-effects model examining the influences on yolk sac absorption of Atlantic salmon alevins (n = 114) originating from females which had spent varying lengths of time in the hatchery (n = 15). Estimates of the magnitude of the effect of each parameter on yolk sac area (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	22.908	0.740	516	30.956	**< 0.001**
14 months	1.569	0.870	11	1.804	0.099
26 months	2.260	2.102	11	1.076	0.305
Developmental time	-11.094	0.405	516	-27.389	**< 0.001**
Opercular beat rate (OBR)	-0.360	0.519	89	-0.694	0.490
Egg Weight	1.060	1.020	89	1.039	0.301
Egg size category	-0.367	0.500	89	-0.735	0.464
14 months x Developmental time	-2.196	0.514	516	-4.270	**< 0.001**
26 months x Developmental time	-4.389	0.610	516	-7.194	**< 0.001**
14 months x OBR	0.085	0.678	89	0.125	0.901
26 months x OBR	1.359	1.102	89	1.234	0.220
14 months x Egg weight	1.499	1.100	89	1.363	0.177
26 months x Egg weight	3.434	1.663	89	2.065	**0.042**
OBR x Egg weight	-0.153	0.401	89	-0.381	0.704
OBR x Developmental time of OBR	0.329	0.427	89	0.771	0.443

c) Results of initial linear mixed-effects model examining the influences on timing of emergence from the gravel of Atlantic salmon alevins (n =2421) originating from females which had spent varying lengths of time in the hatchery (n = 25). Estimates of the magnitude of the effect of each parameter on timing of emergence (β) and its SE (β SE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	2.946	0.018	2275	162.147	**< 0.001**
14 months	0.011	0.012	22	0.854	0.403
26 months	0.015	0.012	2275	1.225	0.221
Egg Weight	-0.024	0.012	116	-1.925	0.057
Egg size category	-0.002	0.005	116	-0.413	0.681
14 months x Egg weight	0.028	0.014	116	1.966	0.052
26 months x Egg weight	0.022	0.013	2275	1.728	0.084

d) Results of initial linear mixed-effects model examining the influences on yolk sac reserves at the time of emergence from the gravel of juvenile Atlantic salmon (n = 1988) originating from females which had spent varying lengths of time in the hatchery (n = 25). Estimates of the magnitude of the effect of each parameter on yolk sac area (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	0.813	0.058	1838	13.992	**< 0.001**
14 months	0.104	0.054	22	1.903	0.070
26 months	0.118	0.052	1838	2.284	**0.023**
Egg Weight	0.046	0.048	116	0.962	0.338
Timing of emergence (DDs)	-0.153	0.010	1838	-15.763	**< 0.001**
Egg size category	-0.016	0.011	116	-1.541	0.126
14 months x Egg weight	0.099	0.054	116	1.827	0.070
26 months x Egg weight	0.019	0.049	1838	0.385	0.701
14 months x Timing of emergence	0.017	0.011	1838	1.547	0.122
26 months x Timing of emergence	-0.002	0.012	1838	-0.173	0.863
Egg weight x Timing of emergence	0.008	0.005	1838	1.730	0.084

e) Results of initial generalised linear mixed-effects model examining the influences on aggressive behaviour at the time of emergence from the gravel of juvenile Atlantic salmon (n = 299) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on aggressive behaviour (β) and its SE (βSE) are indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**z**	**p**
Intercept	1.381	0.153	9.020	**< 0.001**
14 months	-0.444	0.186	-2.388	0.017
26 months	-0.564	0.197	-2.859	0.004
Egg Weight	0.308	0.126	2.445	**0.015**
Emergence timing (DDs)	0.187	0.419	0.446	0.655
Egg size category	0.062	0.148	0.416	0.677
Developmental age (DDs)	-0.604	0.418	-1.446	0.148
Opercular beat rate (OBR)	0.042	0.082	0.508	0.611
Body size (SL)	-0.127	0.111	-1.140	0.254
Temperature	-0.143	0.077	-1.867	0.062
Egg weight x Emergence timing	0.155	0.083	1.866	0.062
Opercular beat rate x Body size	0.053	0.071	0.749	0.454

f) Results of initial linear mixed-effects model examining the influences on opercular beat rate (proxy for metabolic rate) at the time of emergence from the gravel of juvenile Atlantic salmon (n = 299) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on OBR (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	61.770	1.192	274	51.821	**< 0.001**
14 months	0.530	1.485	15	0.357	0.726
26 months	1.158	1.559	15	0.743	0.469
Egg Weight	4.442	0.922	274	4.819	**< 0.001**
Emergence timing (DDs)	5.569	3.159	274	1.763	0.079
Egg size category	0.888	1.081	274	0.821	0.412
Developmental age (DDs)	-7.876	3.133	274	-2.514	**0.013**
Body size (SL)	-3.173	0.812	274	-3.908	**< 0.001**
Temperature	-0.431	0.541	274	-0.797	0.426
Egg weight x Emergence timing	-0.863	0.610	274	-1.415	0.158

g) Results of initial linear mixed-effects model examining the influences on juvenile Atlantic salmon survival released as fry (n = 144) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on survival (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**DF**	**t**	**p**
Intercept	0.076	2.359	107	0.032	0.974
Egg weight	4.289	20.206	12	0.212	0.836
Site 2	-0.791	1.045	107	-0.757	0.451
Site 3	-0.952	1.045	107	-0.911	0.365
Site 4	0.365	1.045	107	0.349	0.727
14 months	-0.654	2.371	12	-0.275	0.787
26 months	-1.348	3.232	12	-0.417	0.684
Time (DPR)	0.014	0.021	107	0.650	0.517
Egg weight x Site 2	5.730	8.471	107	0.676	0.500
Egg weight x Site 3	8.303	8.471	107	0.980	0.329
Egg weight x Site 4	-3.057	8.471	107	-0.361	0.719
Egg weight x 14 months	11.849	20.175	12	0.587	0.568
Egg weight x 26 months	13.110	25.706	12	0.510	0.619
Site 2 x 14 months	-0.208	0.327	107	-0.636	0.526
Site 3 x 14 months	-0.194	0.327	107	-0.593	0.554
Site 4 x 14 months	-0.402	0.327	107	-1.032	0.221
Site 2 x 26 months	-0.095	0.360	107	-0.263	0.793
Site 3 x 26 months	-0.150	0.360	107	-0.417	0.678
Site 4 x 26 months	-0.031	0.360	107	-0.087	0.931
Egg weight x Time (DPR)	-0.087	0.171	107	-0.508	0.613
Site 2 x Time (DPR)	0.001	0.008	107	0.182	0.856
Site 3 x Time (DPR)	$0.830\ e^{-4}$	0.008	107	-0.011	0.991
Site 4 x Time (DPR)	0.002	0.008	107	0.269	0.788
14 months x Time (DPR)	-0.005	0.007	107	-0.777	0.439
26 months x Time (DPR)	0.0002	0.007	107	0.028	0.977

h) Results of linear mixed-effects model examining the influences on body shape of juvenile Atlantic salmon (n = 206) originating from females which had spent varying lengths of time in the hatchery (n = 16). Estimates of the magnitude of the effect of each parameter on body shape (β) and its SE (βSE) are indicated. Degrees of freedom (DF) for each factor are also indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	β	βSE	DF	t	p
PC1					
Intercept	0.014	0.004	175	3.860	**< 0.001**
14 months	-0.010	0.005	13	-2.179	**0.048**
26 months	-0.013	0.004	13	-2.972	**0.011**
Site 2	-0.018	0.006	175	-3.124	**0.002**
Site 3	-0.021	0.005	175	-4.277	**< 0.001**
Site 4	-0.008	0.005	175	-1.592	0.113
Time (DPR)	0.007	0.002	175	2.978	**0.003**
14 months x Site 2	0.012	0.007	175	1.809	0.072
26 months x Site 2	0.011	0.007	175	1.602	0.111
14 months x Site 3	0.011	0.006	175	1.643	0.102
26 months x Site 3	0.011	0.006	175	0.800	0.074
14 months x Site 4	0.005	0.007	175	0.716	0.475
26 months x Site 4	0.014	0.006	175	2.292	**0.023**
14 months x Time (DPR)	-0.002	0.002	175	-0.820	0.413
26 months x Time (DPR)	-0.001	0.002	175	-0.617	0.538
Site 2 x Time (DPR)	-0.007	0.002	175	-3.022	**0.003**
Site 3 x Time (DPR)	-0.005	0.002	175	-1.987	**0.049**
Site 4 x Time (DPR)	0.004	0.002	175	1.691	0.093

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Parameter	β	βSE	DF	t	p
PC2					
Intercept	0.008	0.003	175	2.182	**0.030**
14 months	-0.004	0.004	13	-0.916	0.377
26 months	-0.006	0.004	13	-1.468	0.166
Site 2	-0.002	0.005	175	-0.460	0.646
Site 3	-0.013	0.004	175	-2.887	**0.004**
Site 4	-0.005	0.004	175	-1.189	0.236
Time (DPR)	-0.002	0.002	175	-0.951	0.343
14 months x Site 2	0.003	0.006	175	0.414	0.680
26 months x Site 2	0.003	0.006	175	0.466	0.642
14 months x Site 3	0.007	0.006	175	1.170	0.244
26 months x Site 3	0.005	0.005	175	0.971	0.333
14 months x Site 4	0.0002	0.006	175	0.032	0.975
26 months x Site 4	0.002	0.005	175	0.387	0.699
14 months x Time (DPR)	-0.001	0.002	175	-0.479	0.633
26 months x Time (DPR)	0.001	0.002	175	0.278	0.781
Site 2 x Time (DPR)	0.005	0.002	175	2.160	**0.032**
Site 3 x Time (DPR)	0.005	0.002	175	2.436	**0.016**
Site 4 x Time (DPR)	-0.003	0.002	175	-1.321	0.188

i) Results of initial generalised linear mixed-effects model examining the influences on fluctuating asymmetry in juvenile Atlantic salmon survival released as fry (n = 288) originating from females which had spent varying lengths of time in the hatchery (n = 18). Estimates of the magnitude of the effect of each parameter on fluctuating asymmetry (β) and its SE (βSE) are indicated. P-values falling below the critical α (0.05) are boldfaced.

Parameter	**β**	**βSE**	**Z**	**p**
Intercept	-0.324	0.888	-0.366	0.715
14 months	1.724	1.095	1.575	0.115
26 months	-0.113	1.044	-0.108	0.914
Site 2	1.646	1.267	1.300	0.194
Site 3	-0.185	1.100	-0.168	0.867
Site 4	0.095	1.022	0.093	0.926
Time (DPR)	-1.320	0.510	-2.587	**0.010**
Site 2 x 14 months	-2.983	1.501	-1.987	**0.047**
Site 2 x 26 months	-0.312	1.477	-0.211	0.833
Site 3 x 14 months	-0.955	1.425	-0.670	0.503
Site 3 x 26 months	0.622	1.307	0.476	0.634
Site 4 x 14 months	-1.250	1.398	-0.894	0.371
Site 4 x 26 months	0.055	1.236	0.044	0.965
14 months x Time (DPR)	-0.246	0.483	-0.509	0.611
26 months x Time (DPR)	-0.293	0.425	-0.688	0.491
Site 2 x Time (DPR)	0.379	0.542	0.699	0.485
Site 3 x Time (DPR)	0.291	0.509	0.572	0.568
Site 4 x Time (DPR)	1.132	0.499	2.267	**0.023**

Appendix II. Summary of re-captured fry from four sites in the Taff in 2013 assigned to hatchery crosses. Conf.; Confidence for the trio (offspring, mother and father) detailed as 95% (strict, denoted *) was estimated using CERVUS. Fry only assigned by PAPA are denoted by P; CERVUS by C. Comb.; Combination of alleles used in PAPA (See footnote).

Fish ID	Time (DPS)	Site	Female	Male	Trio loci compared	Trio loci mismatching	Conf.	Comb.
Taff-S1-T1-36	20	Maerdy	F01	M01	10	3	*	1
Taff-S1-T1-52	20	Maerdy	F01	M01	10	2	*	1
Taff-S2-T1-07	20	Clydach	F01	M02	10	2	*	1
Taff-S2-T1-71	20	Clydach	F01	M02	10	1	*	1
Taff-S2-T1-95	20	Clydach	F01	M01	10	1	*	1
Taff-S2-T1-99	20	Clydach	F01	M01	10	0	*	1
Taff-S3-T1-12	20	Aberdare	F01	M01			P	1
Taff-S3-T1-25	20	Aberdare	F01	M02	10	3	*	1
Taff-S3-T1-34	20	Aberdare	F01	M01	9	3	*	1
Taff-S3-T1-46	20	Aberdare	F01	M02	10	1	*	1
Taff-S3-T1-47	20	Aberdare	F01	M02			P	3
Taff-S4-T1-31	20	Penderyn	F01	M02	10	1	*	1
Taff-S4-T1-42	20	Penderyn	F01	M02	8	3	*	1
Taff-S4-T1-75	20	Penderyn	F01	M02	9	2	*	1
Taff-S4-T1-87	20	Penderyn	F01	M01	9	4	*	1
Taff-S4-T1-94	20	Penderyn	F01	M02	10	1	*	1
Taff-S1-T2-01	55	Maerdy	F01	M01	8	2	*	1
Taff-S1-T2-02	55	Maerdy	F01	M02	8	0	*	1
Taff-S2-T2-01	55	Clydach	F01	M02	8	1	*	1
Taff-S3-T2-22	55	Aberdare	F01	M01	8	3	*	1
Taff-S3-T2-47	55	Aberdare	F01	M01	7	2	*	1
Taff-S3-T2-49	55	Aberdare	F01	M02	8	0	*	1
Taff-S3-T2-67	55	Aberdare	F01	M01	8	1	*	1
Taff-S4-T2-46	55	Penderyn	F01	M01			P	5
Taff-S1-T1-43	20	Maerdy	F02	M04	10	2	*	1
Taff-S1-T1-70	20	Maerdy	F02	M03			P	4
Taff-S1-T1-88	20	Maerdy	F02	M03	10	2	*	1
Taff-S3-T1-23	20	Aberdare	F02	M04	10	2	*	1
Taff-S4-T1-80	20	Penderyn	F02	M04	9	1	*	1
Taff-S1-T2-31	55	Maerdy	F02	M03	8	0	*	1
Taff-S1-T2-43	55	Maerdy	F02	M03	8	2	*	1
Taff-S1-T2-50	55	Maerdy	F02	M03	7	1	*	C
Taff-S2-T2-60	55	Clydach	F02	M04	8	1	*	1
Taff-S2-T2-85	55	Clydach	F02	M03	8	2	*	1
Taff-S3-T2-26	55	Aberdare	F02	M03	8	0	*	1

Taff-S3-T2-36	55	Aberdare	F02	M04	8	0	*	1
Taff-S3-T2-83	55	Aberdare	F02	M03	8	1	*	1
Taff-S1-T1-22	20	Maerdy	F03	M05	10	2	*	1
Taff-S1-T1-29	20	Maerdy	F03	M05	10	2	*	1
Taff-S1-T1-30	20	Maerdy	F03	M05	10	1	*	1
Taff-S1-T1-49	20	Maerdy	F03	M05	10	1	*	1
Taff-S1-T1-54	20	Maerdy	F03	M05	10	4	*	1
Taff-S1-T1-80	20	Maerdy	F03	M06	9	4	*	2
Taff-S2-T1-33	20	Clydach	F03	M05	10	2	*	1
Taff-S2-T1-43	20	Clydach	F03	M05	10	4	*	1
Taff-S2-T1-65	20	Clydach	F03	M06	10	2	*	1
Taff-S2-T1-79	20	Clydach	F03	M06	10	1	*	1
Taff-S3-T1-10	20	Aberdare	F03	M06	9	2	*	1
Taff-S3-T1-19	20	Aberdare	F03	M06	10	1	*	1
Taff-S3-T1-58	20	Aberdare	F03	M06			P	1
Taff-S3-T1-81	20	Aberdare	F03	M06	9	2	*	1
Taff-S3-T1-94	20	Aberdare	F03	M06	9	1	*	1
Taff-S4-T1-07	20	Penderyn	F03	M06	10	1	*	1
Taff-S4-T1-11	20	Penderyn	F03	M06	9	0	*	2
Taff-S4-T1-13	20	Penderyn	F03	M06	8	1	*	C
Taff-S4-T1-20	20	Penderyn	F03	M06	8	0	*	C
Taff-S4-T1-36	20	Penderyn	F03	M06	10	1	*	1
Taff-S4-T1-49	20	Penderyn	F03	M06	10	2	*	1
Taff-S1-T2-04	55	Maerdy	F03	M05	8	1	*	1
Taff-S1-T2-60	55	Maerdy	F03	M05	8	2	*	1
Taff-S2-T2-44	55	Clydach	F03	M06	8	1	*	1
Taff-S3-T2-08	55	Aberdare	F03	M06	8	0	*	1
Taff-S3-T2-10	55	Aberdare	F03	M06	8	1	*	1
Taff-S3-T2-38	55	Aberdare	F03	M05	8	0	*	1
Taff-S3-T2-54	55	Aberdare	F03	M06	8	1	*	1
Taff-S3-T2-96	55	Aberdare	F03	M06	8	1	*	1
Taff-S4-T2-35	55	Penderyn	F03	M06	8	1	*	1
Taff-S2-T2-87	55	Clydach	F04	M08	8	2	*	1
Taff-S4-T2-51	55	Penderyn	F04	M08			P	1
Taff-S4-T2-90	55	Penderyn	F04	M07	6	2	*	5
Taff-S1-T1-06	20	Maerdy	F05	M10	10	2	*	2
Taff-S1-T1-12	20	Maerdy	F05	M10	10	4	*	C
Taff-S1-T1-48	20	Maerdy	F05	M09	10	3	*	1
Taff-S1-T1-57	20	Maerdy	F05	M09			P	1
Taff-S1-T1-60	20	Maerdy	F05	M09	10	2	*	1
Taff-S1-T1-83	20	Maerdy	F05	M10	10	2	*	1
Taff-S2-T1-06	20	Clydach	F05	M09	10	4	*	1
Taff-S2-T1-14	20	Clydach	F05	M10	10	1	*	1
Taff-S2-T1-38	20	Clydach	F05	M10	10	1	*	1
Taff-S2-T1-41	20	Clydach	F05	M09	10	2	*	1
Taff-S2-T1-51	20	Clydach	F05	M10	10	1	*	1

Taff-S2-T1-62	20	Clydach	F05	M09	10	2	*	1
Taff-S2-T1-84	20	Clydach	F05	M10	10	2	*	1
Taff-S2-T1-97	20	Clydach	F05	M09	10	4	*	1
Taff-S3-T1-14	20	Aberdare	F05	M09	10	2	*	1
Taff-S3-T1-41	20	Aberdare	F05	M09	10	1	*	1
Taff-S3-T1-43	20	Aberdare	F05	M10	9	1	*	1
Taff-S3-T1-45	20	Aberdare	F05	M10	10	3	*	1
Taff-S3-T1-49	20	Aberdare	F05	M10			P	1
Taff-S3-T1-57	20	Aberdare	F05	M10	10	1	*	1
Taff-S3-T1-73	20	Aberdare	F05	M09	10	2	*	2
Taff-S4-T1-25	20	Penderyn	F05	M10	10	1	*	1
Taff-S4-T1-28	20	Penderyn	F05	M10	8	0	*	C
Taff-S4-T1-32	20	Penderyn	F05	M10	10	2	*	1
Taff-S4-T1-43	20	Penderyn	F05	M10	9	1	*	1
Taff-S4-T1-59	20	Penderyn	F05	M09			P	1
Taff-S4-T1-65	20	Penderyn	F05	M10	8	1	*	1
Taff-S4-T1-86	20	Penderyn	F05	M09	9	2	*	1
Taff-S4-T1-96	20	Penderyn	F05	M10	10	3	*	1
Taff-S4-T1-97	20	Penderyn	F05	M09	9	3	*	2
Taff-S1-T2-27	55	Maerdy	F05	M10	8	3	*	1
Taff-S1-T2-33	55	Maerdy	F05	M10	8	1	*	1
Taff-S2-T2-26	55	Clydach	F05	M10	8	1	*	1
Taff-S2-T2-41	55	Clydach	F05	M10	8	0	*	1
Taff-S2-T2-48	55	Clydach	F05	M10	8	2	*	1
Taff-S2-T2-67	55	Clydach	F05	M10	8	1	*	1
Taff-S2-T2-82	55	Clydach	F05	M09	8	4	*	1
Taff-S2-T2-83	55	Clydach	F05	M09	8	1	*	1
Taff-S3-T2-41	55	Aberdare	F05	M10	8	1	*	1
Taff-S3-T2-62	55	Aberdare	F05	M09	8	1	*	1
Taff-S3-T2-68	55	Aberdare	F05	M10	8	0	*	1
Taff-S3-T2-71	55	Aberdare	F05	M09	8	1	*	1
Taff-S3-T2-72	55	Aberdare	F05	M09	8	1	*	1
Taff-S3-T2-94	55	Aberdare	F05	M09	8	1	*	1
Taff-S4-T2-10	55	Penderyn	F05	M10	8	1	*	1
Taff-S4-T2-18	55	Penderyn	F05	M09	8	1	*	1
Taff-S4-T2-19	55	Penderyn	F05	M09	8	2	*	1
Taff-S4-T2-26	55	Penderyn	F05	M09			P	1
Taff-S4-T2-30	55	Penderyn	F05	M10	8	1	*	1
Taff-S4-T2-34	55	Penderyn	F05	M10	8	1	*	1
Taff-S4-T2-42	55	Penderyn	F05	M10	8	1	*	1
Taff-S4-T2-43	55	Penderyn	F05	M10	7	3	*	1
Taff-S4-T2-45	55	Penderyn	F05	M09			P	1
Taff-S4-T2-50	55	Penderyn	F05	M10	8	1	*	1
Taff-S4-T2-66	55	Penderyn	F05	M10	8	3	*	1
Taff-S1-T1-45	20	Maerdy	F06	M12	10	1	*	1
Taff-S1-T1-79	20	Maerdy	F06	M11	9	3	*	C

Taff-S1-T1-84	20	Maerdy	F06	M12	8	2	*	2
Taff-S2-T1-03	20	Clydach	F06	M11	10	1	*	1
Taff-S2-T1-30	20	Clydach	F06	M12	10	0	*	1
Taff-S2-T1-40	20	Clydach	F06	M11	10	0	*	1
Taff-S2-T1-46	20	Clydach	F06	M12	10	1	*	1
Taff-S2-T1-55	20	Clydach	F06	M12	10	0	*	1
Taff-S2-T1-81	20	Clydach	F06	M12	10	0	*	1
Taff-S2-T1-93	20	Clydach	F06	M11	10	1	*	1
Taff-S3-T1-100	20	Aberdare	F06	M11	10	2	*	1
Taff-S3-T1-42	20	Aberdare	F06	M11	10	2	*	1
Taff-S3-T1-87	20	Aberdare	F06	M12	9	1	*	1
Taff-S3-T1-89	20	Aberdare	F06	M11	10	1	*	1
Taff-S3-T1-90	20	Aberdare	F06	M12	9	1	*	1
Taff-S4-T1-19	20	Penderyn	F06	M12			P	2
Taff-S4-T1-33	20	Penderyn	F06	M11	9	3	*	1
Taff-S4-T1-34	20	Penderyn	F06	M12	10	2	*	1
Taff-S4-T1-37	20	Penderyn	F06	M12	10	0	*	1
Taff-S4-T1-47	20	Penderyn	F06	M12	9	1	*	1
Taff-S4-T1-82	20	Penderyn	F06	M12	8	1	*	1
Taff-S1-T2-06	55	Maerdy	F06	M12	8	1	*	1
Taff-S1-T2-09	55	Maerdy	F06	M12	8	0	*	1
Taff-S1-T2-34	55	Maerdy	F06	M12	8	0	*	1
Taff-S1-T2-61	55	Maerdy	F06	M12	8	1	*	1
Taff-S2-T2-21	55	Clydach	F06	M11	8	1	*	1
Taff-S2-T2-30	55	Clydach	F06	M12	8	1	*	1
Taff-S2-T2-43	55	Clydach	F06	M11	8	0	*	1
Taff-S2-T2-51	55	Clydach	F06	M11	8	1	*	1
Taff-S2-T2-63	55	Clydach	F06	M11	8	0	*	1
Taff-S2-T2-88	55	Clydach	F06	M12	8	0	*	1
Taff-S3-T2-02	55	Aberdare	F06	M12	8	2	*	1
Taff-S3-T2-11	55	Aberdare	F06	M11	8	1	*	1
Taff-S3-T2-29	55	Aberdare	F06	M11	8	1	*	1
Taff-S3-T2-44	55	Aberdare	F06	M12	8	2	*	1
Taff-S3-T2-61	55	Aberdare	F06	M11	8	0	*	1
Taff-S3-T2-65	55	Aberdare	F06	M11	8	1	*	1
Taff-S3-T2-69	55	Aberdare	F06	M11	8	2	*	1
Taff-S3-T2-74	55	Aberdare	F06	M11	8	0	*	1
Taff-S3-T2-99	55	Aberdare	F06	M12	8	2	*	1
Taff-S4-T2-08	55	Penderyn	F06	M12	8	1	*	1
Taff-S4-T2-09	55	Penderyn	F06	M11	8	1	*	1
Taff-S4-T2-23	55	Penderyn	F06	M11	8	1	*	1
Taff-S4-T2-24	55	Penderyn	F06	M12	8	0	*	1
Taff-S4-T2-49	55	Penderyn	F06	M12	8	3	*	1
Taff-S4-T2-78	55	Penderyn	F06	M11	7	2	*	5
Taff-S4-T2-83	55	Penderyn	F06	M12	7	0	*	5
Taff-S4-T2-84	55	Penderyn	F06	M12	7	1	*	5

Taff-S4-T2-89	55	Penderyn	F06	M11	8	1	*	1
Taff-S4-T2-98	55	Penderyn	F06	M12	8	2	*	1
Taff-S1-T1-09	20	Maerdy	F07	M02	10	2	*	1
Taff-S1-T1-21	20	Maerdy	F07	M02	10	2	*	1
Taff-S1-T1-23	20	Maerdy	F07	M01	10	2	*	1
Taff-S1-T1-61	20	Maerdy	F07	M02	10	3	*	1
Taff-S2-T1-59	20	Clydach	F07	M01	10	2	*	1
Taff-S3-T1-01	20	Aberdare	F07	M01	9	3	*	1
Taff-S3-T1-28	20	Aberdare	F07	M01	10	3	*	1
Taff-S3-T1-68	20	Aberdare	F07	M02	10	2	*	1
Taff-S3-T1-76	20	Aberdare	F07	M01	9	2	*	1
Taff-S3-T1-77	20	Aberdare	F07	M02	9	5	*	1
Taff-S3-T1-83	20	Aberdare	F07	M02	10	3	*	1
Taff-S3-T1-85	20	Aberdare	F07	M01	9	2	*	1
Taff-S4-T1-06	20	Penderyn	F07	M01	8	2	*	3
Taff-S4-T1-35	20	Penderyn	F07	M01	10	3	*	1
Taff-S4-T1-68	20	Penderyn	F07	M01	9	2	*	1
Taff-S4-T1-83	20	Penderyn	F07	M02	9	3	*	1
Taff-S1-T2-16	55	Maerdy	F07	M01	8	1	*	1
Taff-S1-T2-37	55	Maerdy	F07	M01	8	0	*	1
Taff-S1-T2-40	55	Maerdy	F07	M01	8	1	*	1
Taff-S1-T2-56	55	Maerdy	F07	M02	8	1	*	1
Taff-S2-T2-91	55	Clydach	F07	M01	7	2	*	C
Taff-S3-T2-50	55	Aberdare	F07	M01	8	0	*	1
Taff-S4-T2-27	55	Penderyn	F07	M01	8	1	*	1
Taff-S4-T2-39	55	Penderyn	F07	M01	8	0	*	1
Taff-S4-T2-69	55	Penderyn	F07	M01	8	2	*	1
Taff-S4-T2-74	55	Penderyn	F07	M01	8	0	*	1
Taff-S4-T2-75	55	Penderyn	F07	M01	8	2	*	1
Taff-S4-T2-82	55	Penderyn	F07	M01	7	1	*	5
Taff-S1-T1-26	20	Maerdy	F08	M03			P	1
Taff-S1-T1-55	20	Maerdy	F08	M04			P	1
Taff-S2-T1-83	20	Clydach	F08	M03	10	3	*	1
Taff-S3-T1-36	20	Aberdare	F08	M03			P	1
Taff-S4-T1-21	20	Penderyn	F08	M03	7	2	*	C
Taff-S1-T2-05	55	Maerdy	F08	M04	8	0	*	1
Taff-S1-T2-44	55	Maerdy	F08	M04	8	1	*	1
Taff-S2-T2-28	55	Clydach	F08	M04	8	2	*	1
Taff-S2-T2-29	55	Clydach	F08	M04	8	2	*	1
Taff-S2-T2-47	55	Clydach	F08	M03	8	0	*	1
Taff-S3-T2-30	55	Aberdare	F08	M03	8	2	*	1
Taff-S3-T2-63	55	Aberdare	F08	M03	8	1	*	1
Taff-S1-T1-50	20	Maerdy	F09	M06	10	3	*	1
Taff-S2-T1-16	20	Clydach	F09	M05	10	4	*	1
Taff-S2-T1-24	20	Clydach	F09	M05	10	3	*	1
Taff-S2-T1-89	20	Clydach	F09	M05	10	3	*	1

Taff-S3-T1-29	20	Aberdare	F09	M05	9	2	*	1
Taff-S3-T1-30	20	Aberdare	F09	M06	9	4	*	1
Taff-S3-T1-32	20	Aberdare	F09	M06	10	4	*	1
Taff-S4-T1-63	20	Penderyn	F09	M06	9	3	*	1
Taff-S1-T2-23	55	Maerdy	F09	M06	8	1	*	1
Taff-S1-T2-55	55	Maerdy	F09	M06	8	1	*	1
Taff-S2-T2-11	55	Clydach	F09	M06	8	2	*	1
Taff-S2-T2-52	55	Clydach	F09	M05	8	0	*	1
Taff-S2-T2-72	55	Clydach	F09	M06	8	1	*	1
Taff-S2-T2-97	55	Clydach	F09	M06	8	2	*	1
Taff-S2-T2-99	55	Clydach	F09	M06	8	3	*	1
Taff-S3-T2-05	55	Aberdare	F09	M06	7	1	*	5
Taff-S3-T2-51	55	Aberdare	F09	M05	8	1	*	1
Taff-S3-T2-56	55	Aberdare	F09	M06	8	2	*	1
Taff-S3-T2-98	55	Aberdare	F09	M06	7	2	*	5
Taff-S4-T2-99	55	Penderyn	F09	M05	8	2	*	1
Taff-S1-T1-15	20	Maerdy	F10	M08	10	4	*	1
Taff-S1-T1-18	20	Maerdy	F10	M08	10	2	*	1
Taff-S1-T1-19	20	Maerdy	F10	M08	10	2	*	1
Taff-S1-T1-24	20	Maerdy	F10	M07	10	2	*	1
Taff-S1-T1-31	20	Maerdy	F10	M08	10	2	*	1
Taff-S1-T1-38	20	Maerdy	F10	M08	10	2	*	1
Taff-S1-T1-44	20	Maerdy	F10	M07	10	2	*	1
Taff-S1-T1-47	20	Maerdy	F10	M07	10	3	*	1
Taff-S1-T1-51	20	Maerdy	F10	M07			P	1
Taff-S1-T1-53	20	Maerdy	F10	M07	10	3	*	1
Taff-S1-T1-78	20	Maerdy	F10	M07	10	3	*	1
Taff-S1-T1-81	20	Maerdy	F10	M07	8	2	*	2
Taff-S1-T1-85	20	Maerdy	F10	M07			P	1
Taff-S1-T1-94	20	Maerdy	F10	M07	10	3	*	1
Taff-S1-T1-99	20	Maerdy	F10	M08			P	1
Taff-S2-T1-08	20	Clydach	F10	M07	10	2	*	1
Taff-S2-T1-15	20	Clydach	F10	M07	10	3	*	1
Taff-S2-T1-18	20	Clydach	F10	M07	10	2	*	1
Taff-S2-T1-21	20	Clydach	F10	M08	10	2	*	1
Taff-S2-T1-22	20	Clydach	F10	M08	10	2	*	1
Taff-S2-T1-25	20	Clydach	F10	M07	10	3	*	1
Taff-S2-T1-27	20	Clydach	F10	M07	10	3	*	1
Taff-S2-T1-34	20	Clydach	F10	M08	10	2	*	1
Taff-S2-T1-44	20	Clydach	F10	M07	10	2	*	1
Taff-S2-T1-49	20	Clydach	F10	M08	10	2	*	1
Taff-S2-T1-50	20	Clydach	F10	M07	10	3	*	1
Taff-S2-T1-54	20	Clydach	F10	M08	10	2	*	1
Taff-S2-T1-57	20	Clydach	F10	M08	10	2	*	1
Taff-S2-T1-61	20	Clydach	F10	M07	10	2	*	1
Taff-S2-T1-67	20	Clydach	F10	M08	10	2	*	1

Taff-S2-T1-68	20	Clydach	F10	M07	10	4	*	1
Taff-S2-T1-69	20	Clydach	F10	M07	10	2	*	1
Taff-S2-T1-77	20	Clydach	F10	M07	10	2	*	1
Taff-S2-T1-88	20	Clydach	F10	M07	10	2	*	1
Taff-S3-T1-04	20	Aberdare	F10	M07	10	3	*	1
Taff-S3-T1-08	20	Aberdare	F10	M07			P	1
Taff-S3-T1-17	20	Aberdare	F10	M07	9	3	*	1
Taff-S3-T1-35	20	Aberdare	F10	M07			P	1
Taff-S3-T1-38	20	Aberdare	F10	M08	10	2	*	1
Taff-S3-T1-44	20	Aberdare	F10	M07	10	3	*	1
Taff-S3-T1-48	20	Aberdare	F10	M07	8	2	*	C
Taff-S3-T1-65	20	Aberdare	F10	M08	10	3	*	1
Taff-S3-T1-70	20	Aberdare	F10	M07	10	3	*	1
Taff-S3-T1-71	20	Aberdare	F10	M07			P	2
Taff-S3-T1-75	20	Aberdare	F10	M07	10	3	*	1
Taff-S3-T1-79	20	Aberdare	F10	M08	9	3	*	1
Taff-S3-T1-86	20	Aberdare	F10	M07	9	2	*	1
Taff-S3-T1-98	20	Aberdare	F10	M08	9	3	*	1
Taff-S4-T1-01	20	Penderyn	F10	M08	10	3	*	1
Taff-S4-T1-03	20	Penderyn	F10	M08	8	2	*	3
Taff-S4-T1-04	20	Penderyn	F10	M07	8	3	*	3
Taff-S4-T1-14	20	Penderyn	F10	M07	9	1	*	1
Taff-S4-T1-24	20	Penderyn	F10	M07	8	1	*	1
Taff-S4-T1-27	20	Penderyn	F10	M07	8	1	*	1
Taff-S4-T1-53	20	Penderyn	F10	M07	8	2	*	1
Taff-S4-T1-57	20	Penderyn	F10	M08	10	2	*	1
Taff-S4-T1-58	20	Penderyn	F10	M07	9	2	*	1
Taff-S4-T1-71	20	Penderyn	F10	M08	10	4	*	1
Taff-S4-T1-72	20	Penderyn	F10	M07	8	2	*	C
Taff-S4-T1-76	20	Penderyn	F10	M07	9	2	*	1
Taff-S4-T1-77	20	Penderyn	F10	M07	9	3	*	1
Taff-S4-T1-78	20	Penderyn	F10	M07	9	2	*	1
Taff-S4-T1-92	20	Penderyn	F10	M07	10	3	*	1
Taff-S4-T1-93	20	Penderyn	F10	M08	10	2	*	1
Taff-S4-T1-98	20	Penderyn	F10	M07	10	4	*	1
Taff-S4-T1-99	20	Penderyn	F10	M07	10	4	*	1
Taff-S1-T2-03	55	Maerdy	F10	M08	8	2	*	1
Taff-S1-T2-21	55	Maerdy	F10	M07	8	2	*	1
Taff-S1-T2-26	55	Maerdy	F10	M07	8	1	*	1
Taff-S1-T2-30	55	Maerdy	F10	M08	8	1	*	1
Taff-S1-T2-42	55	Maerdy	F10	M07	6	2	*	5
Taff-S1-T2-53	55	Maerdy	F10	M07	7	0	*	C
Taff-S2-T2-02	55	Clydach	F10	M08	8	0	*	1
Taff-S2-T2-08	55	Clydach	F10	M07	7	1	*	5
Taff-S2-T2-10	55	Clydach	F10	M08	8	0	*	1
Taff-S2-T2-12	55	Clydach	F10	M07	8	1	*	1

Taff-S2-T2-16	55	Clydach	F10	M08	8	0	*	1
Taff-S2-T2-17	55	Clydach	F10	M07	8	0	*	1
Taff-S2-T2-32	55	Clydach	F10	M07	8	2	*	1
Taff-S2-T2-50	55	Clydach	F10	M07	8	0	*	1
Taff-S2-T2-54	55	Clydach	F10	M08	8	0	*	1
Taff-S2-T2-70	55	Clydach	F10	M07	8	1	*	1
Taff-S2-T2-74	55	Clydach	F10	M07	8	2	*	1
Taff-S2-T2-75	55	Clydach	F10	M08	8	0	*	1
Taff-S3-T2-20	55	Aberdare	F10	M08	8	1	*	1
Taff-S3-T2-23	55	Aberdare	F10	M08	8	1	*	1
Taff-S3-T2-37	55	Aberdare	F10	M08	8	1	*	1
Taff-S3-T2-43	55	Aberdare	F10	M08	8	1	*	1
Taff-S3-T2-66	55	Aberdare	F10	M08	8	1	*	1
Taff-S3-T2-73	55	Aberdare	F10	M07	8	0	*	1
Taff-S3-T2-79	55	Aberdare	F10	M08	7	2	*	5
Taff-S3-T2-85	55	Aberdare	F10	M08	8	3	*	1
Taff-S4-T2-17	55	Penderyn	F10	M08	8	1	*	1
Taff-S4-T2-25	55	Penderyn	F10	M07	8	1	*	1
Taff-S4-T2-38	55	Penderyn	F10	M07	8	2	*	1
Taff-S4-T2-41	55	Penderyn	F10	M07	8	1	*	1
Taff-S4-T2-55	55	Penderyn	F10	M07	8	1	*	1
Taff-S4-T2-88	55	Penderyn	F10	M07	7	1	*	1
Taff-S4-T2-91	55	Penderyn	F10	M07	8	2	*	1
Taff-S1-T1-04	20	Maerdy	F12	M11	10	4	*	2
Taff-S1-T1-10	20	Maerdy	F12	M11	10	4	*	1
Taff-S1-T1-14	20	Maerdy	F12	M11	10	4	*	1
Taff-S1-T1-27	20	Maerdy	F12	M12			P	1
Taff-S1-T1-37	20	Maerdy	F12	M11	10	5	*	1
Taff-S1-T1-39	20	Maerdy	F12	M11	10	4	*	4
Taff-S1-T1-40	20	Maerdy	F12	M12	10	4	*	1
Taff-S1-T1-65	20	Maerdy	F12	M12	10	4	*	4
Taff-S1-T1-66	20	Maerdy	F12	M12	10	4	*	1
Taff-S1-T1-74	20	Maerdy	F12	M12	10	4	*	1
Taff-S1-T1-75	20	Maerdy	F12	M12	10	4	*	1
Taff-S1-T1-76	20	Maerdy	F12	M12	10	3	*	1
Taff-S1-T1-82	20	Maerdy	F12	M11	8	3	*	2
Taff-S2-T1-12	20	Clydach	F12	M11	10	5	*	1
Taff-S2-T1-20	20	Clydach	F12	M11	10	5	*	1
Taff-S2-T1-37	20	Clydach	F12	M11	10	4	*	1
Taff-S2-T1-39	20	Clydach	F12	M11	10	3	*	1
Taff-S2-T1-52	20	Clydach	F12	M12	10	4	*	1
Taff-S2-T1-60	20	Clydach	F12	M12	10	3	*	1
Taff-S2-T1-80	20	Clydach	F12	M11	10	4	*	1
Taff-S2-T1-91	20	Clydach	F12	M12	10	4	*	1
Taff-S2-T1-94	20	Clydach	F12	M12	10	4	*	1
Taff-S3-T1-06	20	Aberdare	F12	M11	10	5	*	1

Taff-S3-T1-20	20	Aberdare	F12	M11	9	4	*	1
Taff-S3-T1-54	20	Aberdare	F12	M11	9	2	*	2
Taff-S3-T1-66	20	Aberdare	F12	M11	10	4	*	1
Taff-S3-T1-69	20	Aberdare	F12	M12	10	3	*	1
Taff-S3-T1-78	20	Aberdare	F12	M12	10	3	*	1
Taff-S3-T1-80	20	Aberdare	F12	M12	9	3	*	1
Taff-S3-T1-95	20	Aberdare	F12	M12	9	3	*	2
Taff-S4-T1-100	20	Penderyn	F12	M11			P	1
Taff-S4-T1-40	20	Penderyn	F12	M12	9	4	*	1
Taff-S4-T1-45	20	Penderyn	F12	M11	9	4	*	1
Taff-S4-T1-46	20	Penderyn	F12	M11	9	3	*	1
Taff-S4-T1-48	20	Penderyn	F12	M12	9	3	*	1
Taff-S4-T1-52	20	Penderyn	F12	M12	8	4	*	1
Taff-S4-T1-85	20	Penderyn	F12	M12	8	3	*	1
Taff-S4-T1-89	20	Penderyn	F12	M12	10	5	*	1
Taff-S4-T1-91	20	Penderyn	F12	M11	10	4	*	1
Taff-S1-T2-12	55	Maerdy	F12	M12	7	1	*	5
Taff-S1-T2-29	55	Maerdy	F12	M12	8	2	*	1
Taff-S1-T2-47	55	Maerdy	F12	M11	7	3	*	5
Taff-S1-T2-49	55	Maerdy	F12	M12	8	2	*	1
Taff-S1-T2-51	55	Maerdy	F12	M11	8	1	*	1
Taff-S2-T2-13	55	Clydach	F12	M12	8	3	*	1
Taff-S2-T2-71	55	Clydach	F12	M11	8	1	*	1
Taff-S2-T2-81	55	Clydach	F12	M11	8	2	*	1
Taff-S3-T2-15	55	Aberdare	F12	M12	8	1	*	1
Taff-S3-T2-28	55	Aberdare	F12	M11	8	1	*	1
Taff-S3-T2-31	55	Aberdare	F12	M12	8	3	*	1
Taff-S3-T2-34	55	Aberdare	F12	M11	8	2	*	1
Taff-S3-T2-46	55	Aberdare	F12	M11	8	2	*	1
Taff-S3-T2-52	55	Aberdare	F12	M11	8	1	*	1
Taff-S3-T2-53	55	Aberdare	F12	M12	8	1	*	1
Taff-S3-T2-55	55	Aberdare	F12	M12	8	2	*	1
Taff-S3-T2-58	55	Aberdare	F12	M11	8	2	*	1
Taff-S3-T2-82	55	Aberdare	F12	M12	7	2	*	5
Taff-S3-T2-91	55	Aberdare	F12	M12	8	1	*	1
Taff-S4-T2-57	55	Penderyn	F12	M11	8	2	*	1
Taff-S4-T2-67	55	Penderyn	F12	M11	8	3	*	1
Taff-S1-T1-03	20	Maerdy	F13	M02	8	3	*	C
Taff-S1-T1-08	20	Maerdy	F13	M02	10	3	*	1
Taff-S1-T1-28	20	Maerdy	F13	M02	10	2	*	1
Taff-S1-T1-89	20	Maerdy	F13	M02	10	2	*	1
Taff-S1-T1-96	20	Maerdy	F13	M02	10	3	*	1
Taff-S2-T1-74	20	Clydach	F13	M02	10	2	*	1
Taff-S2-T1-96	20	Clydach	F13	M01	10	3	*	1
Taff-S3-T1-09	20	Aberdare	F13	M02	9	3	*	1
Taff-S4-T1-15	20	Penderyn	F13	M01	8	2	*	1

Taff-S4-T1-44	20	Penderyn	F13	M02	10	4	*	1
Taff-S4-T1-81	20	Penderyn	F13	M02	10	2	*	1
Taff-S1-T2-36	55	Maerdy	F13	M02	8	0	*	1
Taff-S2-T2-19	55	Clydach	F13	M01	8	0	*	1
Taff-S3-T2-03	55	Aberdare	F13	M01	8	0	*	1
Taff-S3-T2-07	55	Aberdare	F13	M01	7	0	*	5
Taff-S3-T2-40	55	Aberdare	F13	M01	8	0	*	1
Taff-S3-T2-70	55	Aberdare	F13	M01	8	0	*	1
Taff-S4-T2-04	55	Penderyn	F13	M01	8	1	*	1
Taff-S1-T1-02	20	Maerdy	F14	M03	10	4	*	1
Taff-S1-T1-07	20	Maerdy	F14	M03	10	3	*	1
Taff-S1-T1-20	20	Maerdy	F14	M03	10	2	*	1
Taff-S1-T1-32	20	Maerdy	F14	M04	10	4	*	1
Taff-S1-T1-33	20	Maerdy	F14	M03			P	4
Taff-S1-T1-46	20	Maerdy	F14	M04	10	4	*	1
Taff-S1-T1-59	20	Maerdy	F14	M03	10	3	*	4
Taff-S1-T1-68	20	Maerdy	F14	M03	10	4	*	1
Taff-S1-T1-69	20	Maerdy	F14	M03			P	1
Taff-S1-T1-71	20	Maerdy	F14	M04	10	2	*	1
Taff-S1-T1-72	20	Maerdy	F14	M03	10	3	*	1
Taff-S1-T1-86	20	Maerdy	F14	M04	10	3	*	1
Taff-S1-T1-97	20	Maerdy	F14	M03	10	4	*	1
Taff-S2-T1-04	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-13	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-28	20	Clydach	F14	M03	10	2	*	1
Taff-S2-T1-42	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-45	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-53	20	Clydach	F14	M03	10	2	*	1
Taff-S2-T1-56	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-70	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-73	20	Clydach	F14	M04	10	2	*	1
Taff-S2-T1-76	20	Clydach	F14	M03	10	2	*	1
Taff-S2-T1-78	20	Clydach	F14	M04	10	3	*	1
Taff-S2-T1-82	20	Clydach	F14	M03	10	3	*	1
Taff-S2-T1-85	20	Clydach	F14	M04	10	2	*	1
Taff-S3-T1-03	20	Aberdare	F14	M04	9	2	*	1
Taff-S3-T1-07	20	Aberdare	F14	M03	10	2	*	1
Taff-S3-T1-11	20	Aberdare	F14	M04	10	4	*	1
Taff-S3-T1-13	20	Aberdare	F14	M03	10	3	*	1
Taff-S3-T1-15	20	Aberdare	F14	M03	10	4	*	1
Taff-S3-T1-16	20	Aberdare	F14	M04	10	2	*	1
Taff-S3-T1-24	20	Aberdare	F14	M03			P	1
Taff-S3-T1-33	20	Aberdare	F14	M04	9	3	*	1
Taff-S3-T1-40	20	Aberdare	F14	M03	10	3	*	1
Taff-S3-T1-50	20	Aberdare	F14	M04	10	2	*	1
Taff-S3-T1-56	20	Aberdare	F14	M03	10	2	*	1

Taff-S3-T1-59	20	Aberdare	F14	M04			P	3
Taff-S3-T1-60	20	Aberdare	F14	M03	10	2	*	1
Taff-S3-T1-61	20	Aberdare	F14	M04	9	2	*	3
Taff-S3-T1-64	20	Aberdare	F14	M04	9	3	*	1
Taff-S3-T1-74	20	Aberdare	F14	M04	10	2	*	1
Taff-S3-T1-84	20	Aberdare	F14	M04	9	3	*	2
Taff-S3-T1-92	20	Aberdare	F14	M03			P	1
Taff-S4-T1-09	20	Penderyn	F14	M03	9	2	*	1
Taff-S4-T1-38	20	Penderyn	F14	M03	10	2	*	1
Taff-S4-T1-39	20	Penderyn	F14	M04	9	2	*	1
Taff-S4-T1-50	20	Penderyn	F14	M04	8	2	*	1
Taff-S4-T1-51	20	Penderyn	F14	M03	8	1	*	1
Taff-S4-T1-54	20	Penderyn	F14	M04	8	2	*	1
Taff-S4-T1-56	20	Penderyn	F14	M04	8	1	*	1
Taff-S4-T1-60	20	Penderyn	F14	M03	9	2	*	1
Taff-S4-T1-61	20	Penderyn	F14	M03	9	2	*	1
Taff-S4-T1-64	20	Penderyn	F14	M04	9	2	*	1
Taff-S4-T1-73	20	Penderyn	F14	M04	9	3	*	1
Taff-S4-T1-79	20	Penderyn	F14	M04	8	1	*	1
Taff-S4-T1-90	20	Penderyn	F14	M04	10	2	*	1
Taff-S1-T2-11	55	Maerdy	F14	M03	8	0	*	1
Taff-S1-T2-18	55	Maerdy	F14	M04	8	1	*	1
Taff-S1-T2-28	55	Maerdy	F14	M03	8	0	*	1
Taff-S1-T2-58	55	Maerdy	F14	M03	7	2	*	5
Taff-S2-T2-03	55	Clydach	F14	M04	8	2	*	1
Taff-S2-T2-05	55	Clydach	F14	M03	8	1	*	1
Taff-S2-T2-23	55	Clydach	F14	M04	8	0	*	1
Taff-S2-T2-31	55	Clydach	F14	M04	6	0	*	5
Taff-S2-T2-49	55	Clydach	F14	M04	8	1	*	1
Taff-S2-T2-61	55	Clydach	F14	M03	8	0	*	1
Taff-S2-T2-62	55	Clydach	F14	M03	6	0	*	C
Taff-S2-T2-90	55	Clydach	F14	M04	8	1	*	1
Taff-S2-T2-98	55	Clydach	F14	M04	8	1	*	1
Taff-S3-T2-04	55	Aberdare	F14	M03	8	1	*	1
Taff-S3-T2-06	55	Aberdare	F14	M03	8	0	*	1
Taff-S3-T2-12	55	Aberdare	F14	M03	8	0	*	1
Taff-S3-T2-14	55	Aberdare	F14	M04	8	1	*	1
Taff-S3-T2-24	55	Aberdare	F14	M04	8	1	*	1
Taff-S3-T2-39	55	Aberdare	F14	M04	8	0	*	1
Taff-S3-T2-48	55	Aberdare	F14	M03	8	1	*	1
Taff-S3-T2-78	55	Aberdare	F14	M04	8	1	*	1
Taff-S3-T2-80	55	Aberdare	F14	M04	8	2	*	1
Taff-S3-T2-89	55	Aberdare	F14	M04	8	0	*	1
Taff-S3-T2-93	55	Aberdare	F14	M03	8	0	*	1
Taff-S4-T2-02	55	Penderyn	F14	M03	8	1	*	1
Taff-S4-T2-15	55	Penderyn	F14	M03	8	1	*	1

Taff-S4-T2-16	55	Penderyn	F14	M04	8	2	*	1
Taff-S4-T2-22	55	Penderyn	F14	M04	8	3	*	1
Taff-S4-T2-54	55	Penderyn	F14	M04	7	3	*	5
Taff-S4-T2-65	55	Penderyn	F14	M03	8	1	*	1
Taff-S4-T2-71	55	Penderyn	F14	M04	8	0	*	1
Taff-S4-T2-72	55	Penderyn	F14	M03	8	3	*	1
Taff-S4-T2-76	55	Penderyn	F14	M03	7	1	*	1
Taff-S4-T2-80	55	Penderyn	F14	M04	7	1	*	5
Taff-S4-T2-96	55	Penderyn	F14	M04	8	2	*	1
Taff-S4-T2-97	55	Penderyn	F14	M03	8	2	*	1
Taff-S1-T1-11	20	Maerdy	F15	M06	10	3	*	1
Taff-S1-T1-16	20	Maerdy	F15	M06	10	3	*	1
Taff-S1-T1-67	20	Maerdy	F15	M06	10	3	*	1
Taff-S1-T1-77	20	Maerdy	F15	M05	8	2	*	2
Taff-S1-T1-95	20	Maerdy	F15	M06	9	3	*	2
Taff-S2-T1-11	20	Clydach	F15	M05	10	2	*	1
Taff-S2-T1-36	20	Clydach	F15	M06	10	3	*	1
Taff-S2-T1-87	20	Clydach	F15	M06	10	3	*	1
Taff-S3-T1-02	20	Aberdare	F15	M05	10	3	*	1
Taff-S3-T1-18	20	Aberdare	F15	M06	9	4	*	1
Taff-S3-T1-27	20	Aberdare	F15	M05	9	3	*	1
Taff-S3-T1-88	20	Aberdare	F15	M06	10	2	*	1
Taff-S3-T1-93	20	Aberdare	F15	M06	9	3	*	1
Taff-S4-T1-41	20	Penderyn	F15	M05	10	4	*	1
Taff-S4-T1-55	20	Penderyn	F15	M06	9	1	*	1
Taff-S4-T1-74	20	Penderyn	F15	M06	8	2	*	1
Taff-S1-T2-13	55	Maerdy	F15	M06			P	1
Taff-S1-T2-48	55	Maerdy	F15	M05	8	2	*	1
Taff-S2-T2-06	55	Clydach	F15	M05	8	1	*	1
Taff-S2-T2-27	55	Clydach	F15	M05	8	1	*	1
Taff-S2-T2-64	55	Clydach	F15	M05	8	1	*	1
Taff-S2-T2-66	55	Clydach	F15	M06	6	2	*	C
Taff-S3-T2-01	55	Aberdare	F15	M05	8	0	*	1
Taff-S3-T2-17	55	Aberdare	F15	M05	8	1	*	1
Taff-S3-T2-25	55	Aberdare	F15	M06	8	1	*	1
Taff-S3-T2-35	55	Aberdare	F15	M06	8	1	*	1
Taff-S3-T2-90	55	Aberdare	F15	M05	8	1	*	1
Taff-S3-T2-97	55	Aberdare	F15	M06	8	2	*	1
Taff-S4-T2-01	55	Penderyn	F15	M05	8	1	*	1
Taff-S4-T2-03	55	Penderyn	F15	M05	8	0	*	1
Taff-S4-T2-05	55	Penderyn	F15	M06	8	0	*	1
Taff-S4-T2-100	55	Penderyn	F15	M05	8	2	*	1
Taff-S4-T2-61	55	Penderyn	F15	M06	8	1	*	5
Taff-S4-T2-73	55	Penderyn	F15	M06	7	1	*	1
Taff-S4-T2-81	55	Penderyn	F15	M06	8	0	*	1
Taff-S1-T1-01	20	Maerdy	F16	M08			P	1

Taff-S1-T1-100	20	Maerdy	F16	M07			P	1
Taff-S1-T1-17	20	Maerdy	F16	M07	10	3	*	1
Taff-S1-T1-25	20	Maerdy	F16	M08	10	4	*	1
Taff-S1-T1-35	20	Maerdy	F16	M08	10	2	*	1
Taff-S1-T1-56	20	Maerdy	F16	M07	10	3	*	1
Taff-S1-T1-73	20	Maerdy	F16	M07	10	3	*	1
Taff-S1-T1-98	20	Maerdy	F16	M07	10	4	*	1
Taff-S2-T1-01	20	Clydach	F16	M07	10	5	*	1
Taff-S2-T1-02	20	Clydach	F16	M08	10	4	*	1
Taff-S2-T1-10	20	Clydach	F16	M07			P	1
Taff-S2-T1-100	20	Clydach	F16	M08	10	3	*	1
Taff-S2-T1-19	20	Clydach	F16	M07	10	3	*	1
Taff-S2-T1-23	20	Clydach	F16	M07	10	4	*	1
Taff-S2-T1-29	20	Clydach	F16	M08	10	3	*	1
Taff-S2-T1-31	20	Clydach	F16	M07	10	2	*	1
Taff-S2-T1-32	20	Clydach	F16	M08	10	3	*	1
Taff-S2-T1-35	20	Clydach	F16	M07			P	1
Taff-S2-T1-47	20	Clydach	F16	M07	10	3	*	1
Taff-S2-T1-48	20	Clydach	F16	M08	10	2	*	1
Taff-S2-T1-58	20	Clydach	F16	M08	10	3	*	1
Taff-S2-T1-66	20	Clydach	F16	M08	10	2	*	1
Taff-S2-T1-75	20	Clydach	F16	M07			P	1
Taff-S2-T1-92	20	Clydach	F16	M07	10	4	*	1
Taff-S2-T1-98	20	Clydach	F16	M08	10	2	*	1
Taff-S3-T1-05	20	Aberdare	F16	M08	10	4	*	1
Taff-S3-T1-21	20	Aberdare	F16	M07			P	1
Taff-S3-T1-22	20	Aberdare	F16	M07	10	4	*	1
Taff-S3-T1-26	20	Aberdare	F16	M08			P	1
Taff-S3-T1-51	20	Aberdare	F16	M07			P	1
Taff-S3-T1-53	20	Aberdare	F16	M08			P	1
Taff-S3-T1-62	20	Aberdare	F16	M08	9	2	*	2
Taff-S3-T1-63	20	Aberdare	F16	M07			P	1
Taff-S3-T1-82	20	Aberdare	F16	M08	10	3	*	1
Taff-S3-T1-91	20	Aberdare	F16	M07	9	2	*	1
Taff-S3-T1-96	20	Aberdare	F16	M07	9	3	*	1
Taff-S3-T1-97	20	Aberdare	F16	M08	9	3	*	1
Taff-S3-T1-99	20	Aberdare	F16	M08	9	4	*	1
Taff-S4-T1-16	20	Penderyn	F16	M08			P	1
Taff-S4-T1-17	20	Penderyn	F16	M08	8	2	*	C
Taff-S4-T1-23	20	Penderyn	F16	M08	8	2	*	1
Taff-S4-T1-26	20	Penderyn	F16	M07	8	3	*	1
Taff-S4-T1-30	20	Penderyn	F16	M08	8	3	*	1
Taff-S4-T1-62	20	Penderyn	F16	M07	8	2	*	1
Taff-S4-T1-66	20	Penderyn	F16	M08	8	2	*	1
Taff-S4-T1-69	20	Penderyn	F16	M07	9	2	*	1
Taff-S4-T1-70	20	Penderyn	F16	M08			P	1

Taff-S4-T1-88	20	Penderyn	F16	M08	8	1	*	1
Taff-S4-T1-95	20	Penderyn	F16	M08	10	3	*	1
Taff-S1-T2-07	55	Maerdy	F16	M08	8	1	*	1
Taff-S1-T2-08	55	Maerdy	F16	M07	8	1	*	1
Taff-S1-T2-10	55	Maerdy	F16	M08	8	2	*	1
Taff-S1-T2-15	55	Maerdy	F16	M08	8	0	*	1
Taff-S1-T2-17	55	Maerdy	F16	M07	8	1	*	1
Taff-S1-T2-19	55	Maerdy	F16	M08	8	1	*	1
Taff-S1-T2-20	55	Maerdy	F16	M07	8	1	*	1
Taff-S1-T2-22	55	Maerdy	F16	M07	8	1	*	1
Taff-S1-T2-59	55	Maerdy	F16	M07	8	2	*	1
Taff-S2-T2-15	55	Clydach	F16	M07	8	1	*	1
Taff-S2-T2-20	55	Clydach	F16	M08	8	3	*	1
Taff-S2-T2-22	55	Clydach	F16	M08	8	3	*	1
Taff-S2-T2-35	55	Clydach	F16	M07	7	1	*	C
Taff-S2-T2-38	55	Clydach	F16	M07	8	0	*	1
Taff-S2-T2-40	55	Clydach	F16	M07	8	1	*	1
Taff-S2-T2-45	55	Clydach	F16	M07	8	1	*	1
Taff-S2-T2-46	55	Clydach	F16	M07	8	1	*	1
Taff-S2-T2-58	55	Clydach	F16	M08	8	1	*	1
Taff-S2-T2-69	55	Clydach	F16	M07			P	1
Taff-S2-T2-89	55	Clydach	F16	M07	8	2	*	1
Taff-S2-T2-92	55	Clydach	F16	M08	8	2	*	1
Taff-S2-T2-94	55	Clydach	F16	M07	8	2	*	1
Taff-S3-T2-100	55	Aberdare	F16	M07	7	2	*	1
Taff-S3-T2-18	55	Aberdare	F16	M08	8	1	*	1
Taff-S3-T2-21	55	Aberdare	F16	M08	8	1	*	1
Taff-S3-T2-32	55	Aberdare	F16	M07			P	1
Taff-S3-T2-45	55	Aberdare	F16	M07	8	3	*	1
Taff-S3-T2-59	55	Aberdare	F16	M08	8	1	*	1
Taff-S3-T2-60	55	Aberdare	F16	M07	8	1	*	1
Taff-S3-T2-64	55	Aberdare	F16	M07	8	0	*	1
Taff-S3-T2-77	55	Aberdare	F16	M07	8	1	*	1
Taff-S3-T2-81	55	Aberdare	F16	M08	8	3	*	1
Taff-S3-T2-88	55	Aberdare	F16	M08	8	2	*	1
Taff-S4-T2-06	55	Penderyn	F16	M07	8	3	*	1
Taff-S4-T2-07	55	Penderyn	F16	M07	8	2	*	1
Taff-S4-T2-11	55	Penderyn	F16	M08	8	2	*	1
Taff-S4-T2-12	55	Penderyn	F16	M08			P	1
Taff-S4-T2-29	55	Penderyn	F16	M07	8	2	*	1
Taff-S4-T2-31	55	Penderyn	F16	M07	8	3	*	1
Taff-S4-T2-32	55	Penderyn	F16	M07	8	3	*	1
Taff-S4-T2-44	55	Penderyn	F16	M07	8	2	*	1
Taff-S1-T1-42	20	Maerdy	F17	M09	10	4	*	1
Taff-S1-T1-62	20	Maerdy	F17	M10	10	2	*	1
Taff-S1-T1-63	20	Maerdy	F17	M09	10	3	*	1

Taff-S1-T1-64	20	Maerdy	F17	M10	10	2	*	1
Taff-S1-T1-90	20	Maerdy	F17	M09	9	3	*	C
Taff-S1-T1-91	20	Maerdy	F17	M09	10	2	*	1
Taff-S2-T1-86	20	Clydach	F17	M10	10	3	*	1
Taff-S2-T1-90	20	Clydach	F17	M10	10	2	*	1
Taff-S3-T1-52	20	Aberdare	F17	M09	10	2	*	1
Taff-S4-T1-67	20	Penderyn	F17	M10	8	3	*	C
Taff-S4-T1-84	20	Penderyn	F17	M10	9	2	*	1
Taff-S1-T2-39	55	Maerdy	F17	M10	8	1	*	1
Taff-S2-T2-96	55	Clydach	F17	M10	8	1	*	1
Taff-S3-T2-16	55	Aberdare	F17	M09	8	1	*	1
Taff-S3-T2-19	55	Aberdare	F17	M09	8	0	*	1
Taff-S3-T2-27	55	Aberdare	F17	M10	8	0	*	1
Taff-S3-T2-33	55	Aberdare	F17	M09	8	0	*	1
Taff-S3-T2-95	55	Aberdare	F17	M10	8	0	*	1
Taff-S4-T2-14	55	Penderyn	F17	M10	8	1	*	1
Taff-S4-T2-20	55	Penderyn	F17	M09	8	1	*	1
Taff-S1-T1-34	20	Maerdy	F18	M12	10	2	*	1
Taff-S2-T1-05	20	Clydach	F18	M12	10	3	*	1
Taff-S2-T1-17	20	Clydach	F18	M12	9	3	*	3
Taff-S2-T1-72	20	Clydach	F18	M12	10	4	*	1
Taff-S3-T1-31	20	Aberdare	F18	M11	9	3	*	1
Taff-S3-T1-67	20	Aberdare	F18	M11	10	3	*	1
Taff-S4-T1-08	20	Penderyn	F18	M11	10	3	*	1
Taff-S4-T1-18	20	Penderyn	F18	M12	8	1	*	1
Taff-S1-T2-24	55	Maerdy	F18	M11	8	1	*	1
Taff-S1-T2-25	55	Maerdy	F18	M12	8	0	*	1
Taff-S1-T2-45	55	Maerdy	F18	M12	8	1	*	1
Taff-S2-T2-07	55	Clydach	F18	M12	8	0	*	1
Taff-S2-T2-24	55	Clydach	F18	M11	8	1	*	1
Taff-S2-T2-53	55	Clydach	F18	M11	8	0	*	1
Taff-S3-T2-13	55	Aberdare	F18	M12	8	0	*	1
Taff-S3-T2-92	55	Aberdare	F18	M11	8	1	*	1
Taff-S4-T2-28	55	Penderyn	F18	M12	8	1	*	1
Taff-S4-T2-37	55	Penderyn	F18	M11	7	1	*	1
Taff-S4-T2-52	55	Penderyn	F18	M12	8	2	*	1
Taff-S4-T2-56	55	Penderyn	F18	M11	8	1	*	1
Taff-S4-T2-70	55	Penderyn	F18	M12	8	0	*	1

1 - Ssa202, SSspG7, Sp2201, SsaD144, Sasa-UBA, Sp2216, Ssa197*, SSsp3016, Sasa-DAA, Ssa171.

2 - SSsp2210, SSspG7, Sp2201, Sasa-UBA, Sp1605, Sp2216, Ssa197*, SSsp3016, Sasa-DAA, Ssa171.

3 - Ssa202, SSspG7, SsaD144, Sasa-UBA, Sp1605, Sp2216, Ssa197*, SSsp3016, Sasa-DAA, Ssa171.

4 - SSsp2210, Ssa202, SSspG7, Sp2201, SsaD144, Sasa-UBA, Sp1605, Sp2216, Ssa197*, SSsp3016.

5- Ssa202, SSspG7, SsaD144, Sasa-UBA, Sp2216, Ssa197*, SSsp3016, Sasa-DAA.