The influence of egg size for the diversification of Arctic charr (*Salvelinus alpinus*) morphs

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Early life-stage diversification in Arctic charr
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Abstract

Intraspecific variation in egg size and its effects on offspring fitness is well documented in fishes. Yet whether or not differential per propagule maternal investment (egg size) can influence phenotypic diversification is little understood. Arctic charr (*Salvelinus alpinus*) are famed for their resource polymorphisms, whereby their degree of sympatric diversification often varies between lakes. Using a gradient of divergence, we aimed to understand how egg size influenced: (1) developmental times, (2) relative expression of genes related to growth and skeletogenesis across early-life stages, (3) craniofacial shape at hatching and first feeding, and (4) the covariance between craniofacial shape and the relative expression of genes related to growth and skeletogenesis. We found morphs to differ in egg size and egg size variation, as well their extent of egg size-mediated effects on offspring gene expression and craniofacial shape. Most striking is the higher expression of genes related to skeletogenesis at eye stage in the weakly diverged Vatnshlíðarvatn silver morph, all of which also correlated with egg size and was the only morph to have egg size-correlated differences in craniofacial shape. These results demonstrate the likely role that canalisation has played in reducing phenotypic variation in offpspring (i.e. gene expression and shape), in concert with egg size-mediated maternal effects, along a gradient of sympatric divergence. This thesis provides novel insight into the potential for early-life stage divergence of gene expression and associated shape change, alongside variation in egg size, to drive phenotypic diversification in the early evolution of resource polymorphism.

Útdráttur

Breytileiki á hrognastærð innan og milli tegunda fiska er umtalsverður og hrognastærð hefur áhrif á afkomu og hæfni afkvæma. Skilningur okkar á því hvaða áhrif eggjastærð getur haft á þroskunarferla afkvæma er aftur á móti takmarkaður. Bleikja (*Salvelinus alpinus*) er þekkt fyrir óvenjumikinn svipgerðarbreytileika og mismunandi afbrigði sem nýta ólík búsvæði og fæðu innan sama stöðuvatnsins eru algeng. Ég athugaði hvort breytileiki í stærð hrogna og afkvæma hefur áhrif þroskunarhraða og tjáningu gena tengdum vexti, beinþroskun og lögun þess hluta höfuðkúpunnar sem tengjast fæðunámi. Þetta var metið í afkvæmum við klak og þegar a þau byrjuðu að éta. Slík tengsl hrognastærðar og fósturþroska voru rannsökuð hjá misaðgreindum afbrigðum í tveim stöðuvötnum (Þingvallavatni og Vatnshlíðarvatni), sem og hjá sjóbleikjustofni (Fljótaá). Genatjáning og þroskunarhraði voru ólík milli afbrigða, en hrognastærð hafði einungis áhrif í einu afbrigði. Þannig var tjáning gena sem hafa áhrif höfuðbeinþroskun hlutfallslega há snemma á þroskaferlinu (augnstig) og jafnframt tengd hrognastærð hjá afkvæmum silfraða-afbrigðisins í Vatnshlíðarvatni; en afbrigðin í vatninu eru minna aðskilin er afbrigðin í Þingvallavatni. Samantekið styðja niðurstöður þá tilgátu að sveigjanleiki í þroska svipgerða, m.a. vegna áhrifa hrognastærðar, minnki eftir því sem samsvæða bleikjuafbrigð eru aðgreindari. Helsta nýnæmi ritgerðarinnar felst í því að sýna hvernig breytileiki í genatjáningu og snemmþroska svipgerða geti skýrt tilurð ólíkra afbrigða innan tegunda og að á fyrstu stigum slíkrar þróunar geti hrognastærð haft áhrif á sveigjanleika þessara þátta.

List of papers

This thesis is based on four papers, one of which has been published whilst the remaining three are manuscripts. In the text, the papers are referred to with their respective numbers as follows:

- Paper I: Samantha V. Beck, Katja Räsänen, Bjarni K. Kristjánsson, Skúli Skúlason, Zophonías O. Jónsson, Camille A. Leblanc. Importance of egg size for phenotypic variation in sympatric and allopatric populations of polymorphic Arctic charr (Manuscript).

- Paper II: Samantha V. Beck, Katja Räsänen, Ehsan P. Ahi, Bjarni K. Kristjánsson, Skúli Skúlason, Zophonías O. Jónsson, Camille A. Leblanc (2019) Gene expression in the phenotypically plastic Arctic charr (*Salvelinus alpinus*): A focus on growth and ossification at early stages of development. *Evolution & Development*, .

- Paper III: Samantha V. Beck, Katja Räsänen, Camille A. Leblanc, Skúli Skúlason, Zophonías O. Jónsson, Bjarni K. Kristjánsson. The importance of family effects for craniofacial shape variation at early life-stages in Arctic charr (*Salvelinus alpinus*) (Manuscript).

- Paper IV: Samantha V. Beck, Katja Räsänen, Bjarni K. Kristjánsson, Skúli Skúlason, Zophonías O. Jónsson, Anett Reilent, Camille A. Leblanc. Egg size, gene expression and early life-stage phenotypic divergence in sympatric and allopatric Arctic charr (*Salvelinus alpinus*) morphs (Manuscript).

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Glossary

Canalisation The ability of a population/morph to maintain a phenotypic trait regardless of any changes in the environment or genotype.

Developmental trajectory The progression of certain developmental traits in a particular entity (species, population or genotype) that can differ between entities due to environmental or genetic factors.

Epigenetics A pattern of phenotypic inheritance that does not stem from variation in the primary sequence of DNA.

Genetic accommodation The process of adaptive genetic changes affecting the phenotype that can also be influenced by environmental fators. Can either result in increased or reduced levels of plasticity.

Genetic assimilation A specific form of 'genetic accommodation' whereby a phenotype that was brought about by an environmental response, becomes insensitive to the original environmental stimulus. Thus resulting in reduced phenotypic plasticity and genetic fixing of a phenotype previously established by a plastic response to the environment.

Maternal effect The influence of the mothers' environment and phenotype on offspring phenotype, in addition to the direct effect of the genes contributed by her

Morph A group within a species that displays a similar phenotype when two or more groups exist.

Plasticity The interaction of genes and environment can lead to certain genotypes producing very different phenotypes in distinct environments, resulting in plasticity. These changes can occur in development, and thus we here use the term 'plasticity' to encompass plastic changes to phenotype both in early development, as well as in later life stages.

Population A group of inter-breeding individuals of the same species inhabiting the same area.

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1 Chapter I

1.1 General Introduction

The loss of biodiversity today is occurring at unprecedented levels, with anthropogenic activities being the primary driver of current species extinction rates (Ceballos et al., 2015), as well as a major evolutionary force (Hendry et al., 2017). Conservation efforts have focused primarily on species that have large ecological effects, especially those that underpin or provide ecosystem services (i.e. that links ecosystem functioning with human welfare; Sandifer et al., 2015). However, it is becoming increasingly acknowledged that intraspecific variation (phenotypic variation within species) can also have a large impact on ecological processes (Raffard et al., 2018), yet it is suggested that a considerable loss of intraspecific genetic diversity is occurring as populations continue to decline (Ceballos et al., 2017). This loss of intraspecific diversity can influence the stability and resilience of ecosystems, as well as the provision of ecosystem services to a similar extent as, if not more than, interspecific diversity (Des Roches et al., 2018). For example, diversification along a benthic-limnetic axis in threespine stickleback (*Gasterosteus aculeatus*) has had strong direct and indirect effects on ecosystem processes, even at a contemporary evolutionary timescale (Harmon et al., 2009; Des Roches et al., 2013; Matthews et al., 2016). Furthermore, an increase in ecological specialisation in polymorphic European whitefish (*Coregonus lavaretus*) can expand the trophic niche of a top predator (Thomas et al., 2017). Evidence for prey-induced changes in predators has also been found in the predatory chain pickerel (*Esox niger*), whereby the increased availability of its prey, the alewife (*Alosa pseudoharengus*), resulted in its sympatric phenotypic diversification (Brodersen et al., 2015). The maintenance of such intraspecific biodiversity can spread the risk of species losses, as numerous populations can buffer against environmental perturbations (e.g. Figge, 2004). Gaining an understanding of the mechanisms and processes generating and/or maintaining intraspecific diversity may provide novel conservation management options to facilitate population persistence as biodiversity continues to decline.

Contemporary evolution (i.e., micro-evolutionary changes that occur in contemporary timescales) can facilitate rapid phenotypic change (Hairston et al., 2005) and buffer populations from local extirpation or extinction in the face of environmental perturbations (e.g. anthropogenic pressures; Stockwell et al., 2003; Palkovacs et al., 2012). One mechanism of rapid phenotypic change are maternal effects, which occur when the mothers' environment and phenotype influences offspring phenotype, jointly with direct genetic effects (Wolf & Wade, 2009). The capacity for maternal effects to influence offspring phenotype via differential maternal investment is an example of transgenerational plasticity, whereby the developmental processes of the offspring is altered based on the mothers' environmental conditions, that can then be passed on to subsequent generations (Uller, 2008). The mothers' phenotype can not only determine in which environment her offspring develops (Shine & Harlow, 1996; Galloway, 2005), but also offspring size (Heath et al., 1999; Hayward & Wingfield, 2004), quality (Reznick et al., 1996; Marshall et al., 2010; Krist, 2011) and even gender (Nager et al., 1999; Sheldon & West, 2004). Furthermore, the environment experienced by the mother can influence variation in her own condition, including variation in growth rate (i.e. body size) and physiological state, the effects of which can be transmitted to her offspring via various cytoplasmic factors (e.g. mRNA, quantity of yolk, epigenetic changes and hormones; Mousseau & Fox, 1998). These can then either directly - via maternal programming - or indirectly - via offsprings' sensitivity to maternally transmitted material - influence offspring development (Mousseau & Fox, 1998). The egg, to which these maternal factors are deposited, can therefore be considered as a form of environment for the developing embryo, as variation in this maternally transmitted material can alter aspects of not only the immediate embryonic development and associated phenotypic changes, but also that of future generations (e.g. Su et al., 1997; Agrawal et al., 1999), facilitating evolution at ecological timescales (Räsänen & Kruuk, 2007).

1.1.1 Egg size and offspring phenotype

The partitioning of maternal resources among offspring within a given female has been suggested to be in part determined by a trade-off between egg size, and therefore offspring size, and female fecundity (Smith & Fretwell, 1974; Roff, 1992; Stearns, 1992; Einum & Fleming, 2000). This trade-off can be influenced by the environment, maternal genetic effects (Wolf & Wade, 2016), as well as any physiological constraints, i.e. limited amount of energy resources for egg production, or limited space due to body size and/or age at maturity (Einum et al., 2004). The environment is therefore an important source of phenotypic variation in the female that can be consequently passed on to her offspring. As such, the influence of maternal effects on the fitness of offspring can be both adaptive (Agrawal et al., 1999; Galloway, 2005) and non-adaptive (Bernardo, 1996; McCormick, 2006). Variation in egg size can also exist within females, either as a result of physiological constraints such as female age and lack of nutrient delivery to eggs connected to smaller blood vessels (Kamler, 1992), or it can be adaptive to produce variable egg sizes in certain environmental conditions, i.e. a bet-hedging strategy (Hutchings, 1997; Einum et al., 2004; Marshall et al., 2008). For example, Koops et al. (2003) found variation in egg size to increase with environmental unpredictability both within and between females of brook charr (*Salvelinus fontinalis*), which was consistent with an 'environmental predictability hypothesis' (whereby the authors predicted that within-female egg size variability would decrease as the environment becomes more predictable). In birds, variation in egg size has been attributed to females adjusting the size of last-laid eggs to modify the effects of asynchronous hatching to prevent starvation in late-hatching nestlings (Maddox & Weatherhead, 2008). Although that study found that varying egg sizes were unlikely to be an adaptive advantage to females, such differences in developmental timing of offspring can have a profound effect on their fitness, depending upon the subsequent environment and competition in which they are exposed to. Variation in egg size can thus have important implications for offspring survival and fitness. Larger offspring often have higher survival and performance in comparison to smaller offspring (Hutchings, 1991; Williams, 1994; Heath et al., 1999; Räsänen et al., 2005; Perez & Munch, 2010). However, if resources are abundant then offspring size is not such an important determinant of offspring fitness (i.e. survival; Jónsson & Svavarsson, 2000; Marshall et al., 2010; Leblanc et al., 2011, 2016). Understanding the complex suite of biotic and abiotic factors that can drive variation in egg size is a challenging research topic, since numerous factors (i.e. maternal phenotype and genotype, life-history strategies, environmental conditions; Kamler, 1992; Roff, 1992; Bernardo, 1996; Hutchings, 1997) act in synergy to influence egg sizes both within and among females.

Egg size variation has important implications for offspring phenotype and fitness (Mousseau & Fox, 1998), with differential deposition of maternal resources altering embryonic development (e.g. Gil, 2003; Giesing et al., 2011), including developmental time (Pepin et al., 1997; Marshall & Bolton, 2007), survival (Bolton, 1991; Régnier et al., 2013), morphology (Krist, 2009; Cogliati et al., 2018) and behaviour (Benhaïm et al., 2003; Lancaster et al., 2010; Leblanc et al., 2011; Segers & Taborsky, 2011). The ability of offspring to alter their developmental processes and phenotypic outcomes in response to different environmental conditions, including per propagule maternal investment, is known as developmental plasticity (Moczek et al., 2011). Phenotypic plasticity is a wider term that is used to describe the effect of environment on trait expression. However, because developmental processes can strongly mediate the expression of phenotypes (West-Eberhard, 2005), I hereafter refer to phenotypic– and developmental– plasticity, under the umbrella of 'plasticity' (Pfennig, 2016).

Phenotypic consequences for the offspring are, therefore, based on maternal provisioning, as well as the environment the offspring develops in. Both within-generation plasticity and epigenetics (Waddington, 1942a) share the property of causing changes in the phenotype and/or gene expression without changes in the DNA sequence (Schlichting & Wund, 2014). Maternal effects also have the potential to alter the plasticity of their offspring, providing an important mechanism for species, populations and individuals to respond rapidly to changing environments (Kaplan & Cooper, 1984; Einum & Fleming, 1999; Doughty, 2002; Galloway, 2005; Räsänen et al., 2005; Pfennig & Martin, 2009; Donelson et al., 2011; Giesing et al., 2011; Segers & Taborsky, 2012; Feiner et al., 2016; Münzbergová & Hadincová, 2017; Penney et al., 2018). Environmentally based phenotypic variants are thus highly intertwined with underlying molecular mechanisms influencing developmental processes, and therefore the question on whether genes act as 'leaders', or 'followers' to plasticity during the evolution of alternative phenotypes, does not always have a straight forward answer (Schwander & Leimar, 2011).

1.1.2 Plasticity and diversification

Variation in developmental processes as a plastic response to stressful environmental conditions can often lead to phenotypic change, which can later be followed by genetic change when selection favours those phenotypes that are most adaptive (West-Eberhard, 2003; Badyaev, 2005). If an environmentally induced trait continues to be expressed despite changes in the environment (i.e. it loses plasticity), the phenotype has become 'canalised' (Waddington, 1942b; Crispo, 2008). The process of selection acting upon any adaptive genetic changes underlying the expression of the novel trait is known as 'genetic accommodation', which can promote either decreased (canalisation; Waddington, 1942b) or increased (i.e. polyphenism) plasticity (Pfennig, 2016). The process of decreasing – or canalising – plasticity of an environmentally induced phenotype so that it become insensitive to changes in the environment, is known as 'genetic assimilation' (Waddington, 1953), a specific form of genetic accommodation (Pigliucci, 2006; Pfennig, 2016).

For clarity, I here define a population as a group of potentially inter-breeding individuals from the same species inhabiting the same area. Whereas a group within a species that displays a similar phenotype – where two or more groups exist – are called 'morphs'. Morphs can be induced by increased intraspecific competition whereby alternative diets result in resource polymorphism, and can be followed by reproductive isolation (reviewed in: Skúlason & Smith, 1995; Smith & Skúlason, 1996). Resource polymorphisms can therefore increase intraspecific diversification as morphs diverge upon different ecological niches/habitats, resulting in phenotypic evolution (Schluter, 2001). The depauperate nature of lakes in the northern hemisphere has fostered the evolution of resource polymorphism in a number of freshwater systems, where vacant niches have provided opportunity for intraspecific diversification, due to the lack of interspecific competition (Pfennig & Pfennig, 2012). The colonisation of post-glacial lakes by anadromous fishes provides an ideal system to examine evolutionary processes by comparing more derived morphs to putative ancestral populations (i.e., the ancestral 'stem'; West-Eberhard, 2003; Wund et al., 2008; Levis & Pfennig, 2016). For example, in the threespine stickleback (*Gasterosteus aculeatus*), marine populations are considered as the ancestral group. When reared on alternative diets (benthic and pelagic), the plasticity of the head and mouth morphology of the marine fish, show similar phenotypic divergence to that of the derived benthic and pelagic morphs (Wund et al., 2008). As such, it has been hypothesised that the ancestral groups are generally more plastic than more derived/canalised morphs (Skúlason et al., 1999), with empirical evidence providing support for this hypothesis (Parsons et al., 2010, 2011, 2014; Levis et al., 2017). Ancestral plasticity in resource-use traits has been detected in many freshwater fishes (Robinson & Wilson, 1994; Wund et al., 2008; Muschick et al., 2011; Parsons et al., 2014, 2016; Comte & Olden, 2017; Passow et al., 2017), suggesting that a 'plasticity-first' evolutionary scenario might be plausible for numerous freshwater fish species (Schwander & Leimar, 2011; Levis & Pfennig, 2016, 2018). Individual specialisation in diet within a population has been proposed as the first step of trait divergence (Bolnick et al., 2003; Kristjánsson & Leblanc, 2018). If these environmentally induced traits become subject to divergent selection, different morphs may evolve, resulting in phenotypic canalisation in more stable environments (Pfennig & Pfennig, 2012). Plasticity of traits is often accompanied by changes in gene expression, whereby more canalised traits also display a loss of plasticity in gene expression (Levis et al., 2017), highlighting the importance of changes in gene expression in mediating adaptive evolution. Plasticity enables rapid responses to environmental change, and although genetic variation is important for the 'evolvability' of a population, this process takes time in comparison to phenotypes induced by plastic responses, including plastic responses through maternal investment in eggs.

Differential maternal investment in eggs of spadefoot toads (*Spea multiplicata*) was found to mediate rapid population divergence (Pfennig & Martin, 2009). The maternal partitioning of resources in *S. multiplicata* contributed to the evolution of resource polymorphism where larger embryos developed into a carnivorous morph, likely to reduce competition in sympatry (Pfennig et al., 2006; Pfennig & Martin, 2009; Martin & Pfennig, 2010). Such variation in egg/offspring size can result in individuals having to optimise their energy acquisition and behaviour, potentially driving individual specialisation especially when food resources are scarce (Bolnick et al., 2003; Cucherousset et al., 2011). For example, differential energy allocation towards somatic growth and bone development has been found during early embryonic development in different morphs of Arctic charr (*Salvelinus alpinus*; Eiríksson et al., 1999). Furthermore, variation in egg size in this species was found to promote differences in feeding behaviour, even when food resources were abundant (Leblanc et al., 2011). Maternal effects have been seen to mediate adaptive divergence between populations in a number of organisms, such as: birds (Badyaev, 2002), *Drosophila* (Eizadshenass & Singh, 2015; but see, Markov et al., 2016), amphibians (Pfennig & Martin, 2009) and fish (Thorn & Morbey, 2018).

Despite evidence for the importance of maternal effects in mediating population divergence (see references above), as well as changes in gene expression underlying divergence of different morphs (Ahi et al., 2014; Ahi, 2016; Levis et al., 2017; Schneider & Meyer, 2017), as to my knowledge, no other studies have yet linked egg size-correlated changes in gene expression together with associated changes in trophic morphology. Beside my research, only one study has thus far found a link between egg size and gene expression in fishes (but see Ahi *et al.,* 2018). In the mouthbrooding cichlid (*Simochromis pleurospilus*), Segers et al. (2012) found the expression levels of the growth hormone receptor (*GHR*) to be higher in larger cichlid embryos than in smaller embryos - originating from large and small eggs, respectively - a pattern which was then later reversed after hatching.

Differential distribution of maternal mRNAs that are deposited in the egg can be a consequence of differential environmental conditions experienced by the mother (e.g. Crofton et al., 2018). These differences in mRNA deposits can govern early cell division and, in turn, can have the potential to influence embryonic development. Differences in embryonic gene expression have been linked to the evolution of resource polymorphism in Arctic charr (Ahi et al., 2014). Nevertheless, despite the collective evidence suggesting the importance of egg size for offspring fitness and performance, especially in fishes and amphibians, our understanding of the role that egg size might play in the diversification of populations is only recently coming into light (Benhaïm et al., 2003; Pfennig et al., 2006; Leblanc et al., 2011; Cogliati et al., 2018; Ahi et al., 2018; Beck et al., 2018; Self et al., 2018; Thorn & Morbey, 2018; the present research).

Arctic charr are famed for their plasticity, and, as a result of anadromous ancestral populations colonising numerous lakes, have resulted in the parallel evolution of similar phenotypes, often along a benthic-pelagic axis (Skúlason et al., 1999; Kapralova et al., 2011). The degree by which sympatric morphs have diverged from each other varies across lakes, with some morphs having subtle divergence both phenotypically and genetically, e.g. the two morphs ('brown' morph, and 'silver' morph) from lake Vatnshlíðarvatn in Iceland (Gíslason et al., 1999; Jónsson & Skúlason, 2000), whilst lake Þingvallavatn in Iceland has one of the best documented and most extreme cases of sympatric polymorphism, with four discrete morphs: one small and one large benthivorous morph with subterminal lower jaws; and one planktivorous and one piscivorous morph, both pelagic, with evenly protruding jaws (Snorrason et al., 1994). The morphs also differ in life-history characteristics with the larger benthic and piscivorous morphs maturing later than the two smaller morphs (Jonsson et al., 1988). Variation in the feeding apparatus reflects differences in diets and habitat utilisation, which can be due to genetic differences (Skúlason et al., 1989), as well as phenotypic plasticity (Adams & Huntingford, 2004; Klemetsen, 2010; Parsons et al., 2011). The brown morph from the weakly diverged Vatnshlíðarvatn population displays high variation in egg size compared to other morphs of Arctic charr (Beck - *Paper I*). In contrast, the more diverged morphs from lake Þingvallavatn display lower egg size variation within morphs, but have larger egg sizes compared to the Vatnshlíðarvatn morphs (Beck - *Paper I*). Such variation in egg size among sympatric morphs has not yet been examined as a mechanism for influencing evolutionary diversification in Arctic charr (but see Leblanc et al., 2011, 2016). Nevertheless, substantial evidence exists that demonstrates differences in feeding behaviour and mobility (Benhaïm et al., 2003; Leblanc et al., 2011), developmental rates and energy allocation (Eiríksson et al., 1999), energy content and yolk depletion rate (Leblanc et al., 2016) of offspring from different sized eggs, some of which can persist until maturity (Bjorklund et al., 2003). As such, to fully understand the influence of egg size on phenotypic evolution (including changes in gene expression), one must examine how such variation changes across early development at multiple developmental stages. For example: 1) before the switch to zygotic gene expression in the recently fertilised egg (Kane & Kimmel, 1993; Nagasawa et al., 2013); 2) at the organogenesis phase where the choroid of the eye becomes visible through pigmentation (Boyd et al., 2010); 3) once the embryos have hatched but still rely upon endogeneous nutrition (Kamler, 1992); and 4) once the free-swimming embryos have absorbed most of their yolk and transition to feeding on external food sources. The detection of variation in developmental processes between offspring during these developmental time points, especially related to differences in egg size from an ancestral stem to morphs that vary along a gradient of sympatric divergence, can thus provide a novel insight into the evolution of phenotypic variation in sympatric diversification.

1.2 Aims and objectives

The overall aim of this thesis is to understand whether variation in egg size could influence the evolution of resource polymorphism in Arctic charr populations that contain morphs that vary along a gradient of sympatric divergence. I approach this in four different chapters that have the following objectives:

(I) to describe variation in egg size and timing of early developmental stages across multiple populations of Arctic charr that vary in their degree of sympatric morph divergence.

(II) to identify egg/offspring size-correlated gene expression across four developmental stages in a single Arctic charr morph with the most variation in egg size.

(III) to assess the influence of egg/offspring size on craniofacial morphology at hatching and first feeding in a single Arctic charr morph, having the most variable egg sizes.

(IV) to evaluate and compare egg/offspring size-correlated gene expression and associated craniofacial variation in five morphs of Arctic charr, ranging from the least diverged ancestral anadromous population to highly diverged sympatric morphs within populations.

In the first chapter, I asked the following questions:

1. Does egg size and egg size variation differ along a gradient of morph divergence?

2. Does egg size influence developmental rates and size of offspring?

The variability of egg sizes was documented in an anadromous population and six Arctic charr morphs across Iceland. This chapter was essential as it provides a foundation for which all my subsequent studies are based. Egg size, egg size variation, timing of hatching and timing of first feeding were measured in each morph. Means of each trait per female were analysed using linear models, characterising both absolute (maternal investment) and residual egg size (after accounting for female fork length) and whether egg size can influence timing of certain developmental stages. Results showed that in lakes where weakly diverged morphs could be found, there was higher egg size variation but overall smaller eggs in comparison to the lakes where morphs were more highly diverged. Residual egg size variation showed a decline as morphs become more diverged, with the assumed ancestral anadromous population having the highest variation in egg size after accounting for female size differences. Timing of hatching and first feeding was not influenced by egg size, but egg size did influence size at first feeding with offspring from larger eggs feeding at a larger size in a more diverged morph compared to weakly diverged morphs. These results suggest a role for egg size in providing the initial variation in developmental trajectories of offspring, especially in relation to food availability, which may facilitate the evolution of resource polymorphism.

In the second chapter, I studied a (weakly diverged) morph (Vatnshlíðarvatn brown) with high absolute variation in egg size, to ask:

1. Does egg/offspring size influence the expression of genes involved in growth and skeletogenesis?
2. Does gene expression vary across developmental stages?

A total of 14 genes were selected: six genes related to skeletogenesis were chosen based on their differential expression in the developing heads of Arctic charr morphs (Ahi et al., 2014), whilst eight growth-related genes were selected from the literature based on their connection with egg/offspring size. All gene expression results were relative to two housekeeping genes (and are referred to simply as 'gene expression' throughout for brevity). Whole embryos were used for qPCR analyses at three developmental stages: 1) post-fertilisation, 2) eye stage, 3) hatching stage, whilst only the head was used for first feeding stage (4), as the embryos were too large. Gene expression was found to be highly dynamic, varying across all four developmental stages. Only at post-fertilisation stage were mRNA transcripts undetected for *Ets2* and *Star,* indicating that maternal deposition of these two genes were either absent, or too little to be detected by qPCR. Results found little support for egg/offspring size-correlated gene expression with only three out of 14 genes correlating with egg/offspring size: *Mmp9* at post-fertilisation, *Sgk1* and *Star* at hatching stage. Of these three genes, the expression of *Sgk1* was higher in larger offspring, whilst expression of *Mmp9* and *Star* was non-linear in relation to offspring size. Importantly, these three genes are also associated with craniofacial divergence of benthic and limnetic Arctic charr morphs (Ahi et al., 2014). These results indicate that early life-stage variation in gene expression, concomitant to maternal effects, can influence developmental plasticity and potentially the evolution of resource polymorphism in fishes.

In the third chapter, I asked the following questions:

1. Does egg/offspring size influence internal craniofacial morphology of developing Arctic charr embryos?

2. How does internal craniofacial morphology change along early development?

Using an acid-free double-staining protocol, bone and cartilage of offspring from Vatnshlíðarvatn brown were stained, a morph with a wide range of egg sizes (see *Paper I*). Bone and cartilage development of Arctic charr craniofacial structures was characterised at two developmental stages (hatching and first feeding) by selecting 17 fixed and 54 semi-landmarks (homologous across both developmental stages) for geometric morphometric analysis. Principal component analysis (PCA) and Procrustes analysis of variance (ANOVA) were conducted to determine to what extent shape varied across developmental stages, while accounting for family origin, and also whether egg/offspring size influenced craniofacial shape within each developmental stage. Shape did not differ between hatching and first feeding, but because offspring size differed considerably between hatching and first feeding, analyses were also performed within developmental stage. Offspring size had no effect on shape in either developmental stage, and family only had a significant impact on shape at time of first feeding. These results show that offspring size in the Vatnshlíðarvatn brown morph does not affect craniofacial morphology, but family identity has a significant effect on important craniofacial trophic structures at first feeding. These results may suggest that the mechanical constraint of the egg on the embryo may limit visible differences in craniofacial morphologies upon hatching, but such differences may develop or become apparent between hatching and first feeding where offspring from different families showed differences in craniofacial shape. These results thus highlight the importance of considering family effects, and not only body size, of the offspring when examining morphologies of Arctic charr at early life-stages.

Finally, in the fourth chapter, I asked whether:

1. more derived morphs (relative to the putative ancestral anadromous population) had less variation (and thus less covariance) in shape, relative gene expression and less connection with egg size?

2. more derived morphs were less similar to the ancestral population?

3. weakly diverged sympatric morphs were more similar in shape and gene expression in comparison to the highly diverged sympatric morphs?

To answer these questions, an anadromous population and four morphs of Arctic charr (two sympatric pairs from Lakes Vatnshlíðarvatn and Þingvallavatn) were studied to determine whether offspring size and gene expression could be linked to craniofacial shape at hatching and first feeding using qPCR analyses and geometric morphometrics respectively, at a family level. Procrustes ANOVAs were used to assess shape differences between morphs using planned contrasts between sympatric pairs, as well as comparing shape differences to the assumed ancestral anadromous Fljótaá. At hatching, the two Vatnshlíðarvatn morphs significantly differed in shape, whilst only Þingvallavatn planktivorous differed from the ancestral Fljótaá (which was lost after accounting for differences due to allometry, i.e. shape change due to size). At first feeding however, the only differences in planned contrasts were found between the two morphs of Þingvallavatn (both before and after accounting for allometry).

Gene expression was examined within morphs, as well as among morphs, across all four developmental stages. Size had a strong effect on gene expression throughout development for Vatnshlíðarvatn silver, with the most genes being positively correlated to egg size at eye stage. Furthermore, this morph also had much higher relative expression of genes related to skeletogenesis at eye stage in comparison to all other morphs. A single growth-promoting gene was undetected by qPCR at post-fertilisation stage for all morphs except for Þingvallavatn planktivorous. Vatnshlíðarvatn brown, Vatnshlíðarvatn silver and Fljótaá showed size-correlated expression of a gene involved in bone remodelling at post-fertilisation. However, only a small number of individuals had transcripts that were detected in Vatnshlíðarvatn silver and Fljótaá at post-fertilisation, and thus the observed correlation might be an artefact of small sample size. Gene expression at each developmental stage was significantly different between all morphs, including the anadromous population.

Partial least squares (PLS) analyses were then used to assess patterns of covariances between gene expression and craniofacial shape. I first examined if shape at hatching and shape at first feeding covaried across all morphs. Then, within each developmental stage I examined the covariance between craniofacial shape within developmental stage and gene expression. Only hatching stage was found to have covariance between shape and gene expression. Finally, examining covariance within each morph, only gene expression in the weakly diverged Vatnshlíðarvatn silver morph was found to covary with shape.

Using Procrustes ANOVA, I found no difference in craniofacial shape at either hatching stage or first feeding across morphs due to egg size. Within morphs, however, I found that egg size had a significant effect on craniofacial shape in the weakly diverged silver morph from lake Vatnshlíðarvatn, with larger eggs producing offspring with a more bluntened snout in comparison to smaller conspecifics. This morph also drove much of the covariance found between gene expression and craniofacial shape in genes related to skeletogenesis at hatching, and genes related to growth a first feeding.

Overall, results found more derived morphs to have less variation in relative gene expression, but are more *similar* to the ancestral population. Weakly diverged morphs were more different than the strongly diverged morphs in the number of genes differentially expressed, but not for craniofacial shape differences. Egg size had the strongest effect on the weakly diverged and least derived Vatnshlíðarvatn silver morph. Evidence from this study suggests that phenotypic changes among Arctic charr morphs - i.e. changes in gene expression as well as shape – vary across a gradient of divergence, with maternal effects in a more weakly diverged and less derived morph having a larger influence on phenotype than more strongly diverged and more derived/canalised morphs. Maternal effects should therefore be considered in future studies attempting to disentangle the complex processes underlying the evolution of resource polymorphisms.

1.3 General discussion

1.3.1 Patterns of egg size variation

By studying an anadromous population and six morphs of Arctic charr that differ in their degree of sympatric divergence (in comparison to a putative ancestral population), I was able to determine the extent to which egg size and egg size variation changes as sympatric morphs become more diverged, or more specialised (i.e. derived). I found that more diverged and more derived morphs invested in larger eggs, hatched later, and that variation in egg size (after accounting for female length) decreased with increasing divergence – possibly reflecting canalisation of phenotypic variation in more diverged, and derived morphs (Parsons et al., 2010, 2011). The production of larger eggs in these morphs can also be indicative of more adverse environmental conditions, whereby a conservative bet-hedging strategy (i.e. fewer but larger eggs) might be more advantageous (Einum & Fleming, 2004). The morphs that showed the smallest egg sizes were also the only morphs that migrate to spawn (Fljótaá and Vatnshlíðarvatn silver), thus suggesting that environmental conditions favour smaller eggs in more fluvial environments, or that energy spent by migrating females was at the expense of reduced reproductive investment per egg (Braun et al., 2013). The morphs at the weakest end of the divergence spectrum (Fljótaá, Vatnshlíðarvatn silver and Vatnshlíðarvatn brown) showed the most variation in egg size. It has been shown that females that produce larger eggs also tend to produce more consistently sized eggs (Koops et al., 2003; Feiner et al., 2016). The availability of food resources can also be an important determinant for egg size (Hutchings, 1991; Kamler, 1992; Einum & Fleming, 1999; Koops et al., 2003), yet how each of the lakes studied here differ in food availability has not yet been studied. The shallow nature of lake Vatnshlíðarvatn likely limits the variety and availablility of prey for the two weakly divergent morphs (brown and silver), and may also influence egg size based on temperature fluctuations in this lake. Although year-round temperatures were not consistently recorded for all populations, temperature loggers are currently in place at all spawning grounds of morphs studied here to test for temporal differences in temperature, and whether the observed patterns of egg size variation seen here can be related to changes in temperature. Environmental heterogeneity can drive variation in egg size between different populations/morphs, whereby certain sized eggs may be more adaptive than others, and thus becomes canalised so that the phenotypic trait is maintained regardless of any change in the environment or genotype. Comparatively, in more heterogeneous environments, retaining certain degrees of plasticity can be more advantageous in order to respond to changing environmental conditions. Egg size variation may act as a proxy for determining the extent to which plasticity in developmental processes may be required to respond to certain environmental conditions between different morphs. Thus variation in egg size between morphs may be indicative of their degree of canalisation. Furthermore, given the high heritability of egg size in salmonids (Su et al., 1997), as well as its interaction with the environment, egg size-mediated maternal effects are likely to provide a powerful source of variation in offspring phenotype that can be transmitted across generations. Understanding whether patterns of egg size variation between morphs are due to the environment or to canalisation is difficult to disentangle without obtaining temperature data from the wild. Nevertheless, we here provide a strong foundation for future studies wishing to elucidate the mechanisms and processes underlying intraspecific biodiversity in post-glacial lakes in such a well-suited study system.

1.3.2 Morph differences in gene expression and their connection to size

Dynamic changes in relative gene expression were prominent among all five morphs of Arctic charr across all early-life stages. In particular, the planktivorous morph from lake Þingvallavatn had high expression of two genes at post-fertilisation that were not detected in any other population/morph. Maternally derived mRNAs can be crucial for early patterning of embryonic development, thus altering developmental pathways and early morphogenesis (Dworkin & Dworkin-Rastl, 1990). Such differences in gene expression at post-fertilisation may therefore result in large differences in offspring phenotypes via changes in developmental processes and formation of novel developmental pathways (Badyaev, 2008). At eye stage, the silver morph from lake Vatnshlíðarvatn had an almost two-fold increase in relative gene expression in all genes related to skeletogenesis, all of which were also correlated with egg size. The interaction of gene expression with egg size during early development may provide an important source of novelty that could be selected upon in certain environmental conditions. Nevertheless, up until very recently (Ahi et al., 2018), only one other study has documented egg size-correlated gene expression, where larger eggs were found to have higher expression of a growth hormone receptor (a pattern which later reversed after hatching to facilitate compensatory growth; Segers et al., 2012). Here I found every population/morph to have some degree of egg size-correlated expression of genes related to growth and skeletogenesis. The pattern of which is very variable, and it is often intermediate-sized eggs that have lower or higher expression of genes, but none of the morphs show such a high number of egg size-correlated expression of genes than that found in the silver morph from lake Vatnshlíðarvatn. Such patterns can be explained in the context of canalisation, whereby the morphs from lake Vatnshlíðarvatn show very subtle evidence of phenotypic and genetic divergence (Gíslason et al., 1999), and also show more plasticity than more diverged and thus more canalised sympatric morphs (Parsons et al., 2010, 2011). The lack of a clear benthic/pelagic habitat in lake Vatnshlíðarvatn could also be constraining the evolution of these two morphs, that also often overlap in diet (Jónsson & Skúlason, 2000), thus the lack of environmental stability can promote the persistence of phenotypic variation observed both in egg size variation and gene expression in these two morphs. Differences in life histories may play an important role in maintaining the current levels of divergence between the two morphs from lake Vatnshlíðarvatn, as has also been acknowledged in the diversification of benthic and limnetic sticklebacks (Baker et al., 2005).

1.3.3 Craniofacial shape, egg size and their covariance with gene expression

Using the morph with the most variable absolute egg sizes (Vatnshlíðarvatn brown), I found no effect of offspring size on craniofacial shape when examining such patterns at an individual level. Instead, however, I found a strong family effect in offspring craniofacial shape at first feeding (but not at hatching), suggesting that females have the potential to influence dietary intake of offspring based on differences in the trophic apparatus, which is known to be associated with the evolutionary divergence of sympatric morphs as individuals specialise upon different resources (i.e. resource polymorphisms; Skúlason & Smith, 1995; Smith & Skúlason, 1996; Adams et al., 1998; Alekseyev et al., 2002; Bolnick et al., 2003; Ornelas-García et al., 2018). I hypothesise that the lack of such a pattern at hatching stage could be attributable to the release of the embryo from the constraint of the egg, thus permitting differences in developmental trajectories between offspring so that by the time first feeding occurs, such differences can be recognisable. However, such a hypothesis remains to be tested.

When examining differences between morphs, I found the two morphs from lake Vatnshlíðarvatn to differ in craniofacial shape at hatching, a pattern which then disappeared after accounting for differences due to size (i.e. allometry). As for first feeding, the two morphs from lake Þingvallavatn differed in craniofacial shape both before and after accounting for allometry. It is likely that the overlap in diet between the two morphs from lake Vatnshlíðarvatn weakens any signature of shape differentiation in trophic structures required for feeding. However, the variation in trophic structures early in development does exist, and – given abundant resources – I hypothesise that these two morphs would rapidly diverge based on differences in life-history strategies, as well as standing phenotypic variation caused in part by maternal effects, as seen in this study. To provide further evidence towards their lack of canalisation in developmental processes, my results show that the craniofacial shape of Vatnshlíðarvatn silver – as well as demonstrating a substantial number of genes related to skeletogenesis, the expression of which is correlated with egg size – was also correlated with changes in egg size (based on family means), with larger eggs producing offspring with a more bluntened snout than those smaller conspecifics. As suggested above, changes in the shape of trophic apparatuses can have substantial impacts on feeding opportunities, particularly if such shape changes influences mouth gape of offspring (Knutsen & Tilseth, 1985). Furthermore, Vatnshlíðarvatn silver also drove much of the covariance between the relative expression of growth-related genes and craniofacial shape of offspring at first feeding. Such egg size-mediated maternal effects in Vatnshlíðarvatn silver for both craniofacial shape and gene expression, as well as their covariance, suggests that this more generalist and less derived morph retains much more plasticity than its more derived (and thus more canalised) sympatric brown morph. Although a previous study has documented increased canalisation in the more derived brown morph from lake Vatnshlíðarvatn (Parsons et al., 2010), no link has yet been made to the role of maternal effects in mediating such complex diversification processes.

1.3.4 The influence of egg size on the evolutionary diversification of Arctic charr

The overall objective of this thesis was to understand the influence of egg size on the evolutionary diversification of Arctic charr by examining its effects on developmental times and changes in the relative expression of genes related to growth and skeletogenesis, as well as craniofacial shape. Collectively, my results found some evidence for egg/offspring size to influence either gene expression or craniofacial shape across an anadromous population and five morphs of Arctic charr at early-life stages. Although the differential distribution of maternal resources can be an important generator of evolutionary change (Räsänen & Kruuk, 2007; Badyaev, 2008), the lack of strong egg/offspring size-correlated patterns may reflect the release of any selective pressures on offspring reared in laboratory conditions where the environment is kept stable. As such, phenotypic differences stemming from variation in per propagule maternal investment may not become visible until later in development, e.g. after exposure to different diets. There does, however, seem to be substantial differences in the extent to which egg size-mediated maternal effects can influence offspring phenotype between populations/morphs, with offspring from Vatnshlíðarvatn silver exhibiting considerable variation in craniofacial morphology. It has been suggested that females in stressful environments produce offspring with a greater range of variation in skeletal structures (Badyaev, 2005). Movement of offspring during development can be a source of stress on skeletal structures and cause the alteration of certain developmental processes (e.g. Hu & Albertson, 2017). Resulting differences in phenotypes may provide a source of novelty that could potentially play an important role in sympatric diversification. By examining offspring development in response to environmental stressors, i.e. diet availability, one can elucidate how maternal effects – such as those seen in Vatnshlíðarvatn silver – can fine-tune developmental processes to match current environmental conditions. If maternal effects can consistently match the offspring environment, then this epigenetic source of variation may eventually become genetically accommodated in the offspring, facilitating rapid evolutionary diversification (Badyaev, 2008). However, it must be noted that we do not account for the influence of the offsprings' own genes in influencing phenotypic variation (i.e. egg size, craniofacial shape and gene expression). Furthermore, the counfounding nature of female size and population/morph identity makes it difficult to disentangle the effect of both on the phenotype of the offspring. As such, we highlight the importance of examining individuals within a single morph to determine the extent that family effects may play on generating phenotypic variation in offspring.

In conclusion, this research found that the extent of egg size-mediated maternal effects differed between populations/morphs, with the least diverged (sympatrically) and least derived morph (compared to the putative ancestral population) having considerable egg size-correlated effects on offspring gene expression and morphology. The characterisation of egg size and associated phenotypic effects in offspring provides a valuable insight into studies interested in the origin and maintenance of intraspecific diversity in freshwater fishes in the northern hemisphere. Arctic charr provide an ideal model system to examine the role of egg size in influencing evolutionary diversification due to their remarkable phenotypic plasticity and degree of sympatric diversification within lakes. As to my knowledge, this study is the first of its kind to examine how egg size could play a role in the diversification of fishes through correlative effects in gene expression and craniofacial shape.

These results bring to light the potential importance of maternal effects, such as egg size, and their role in the very early generation of phenotypic variation in wild populations of Arctic charr. I provide a baseline documenting the differences in egg/offspring size between sympatric morphs, allopatric morphs and family identity, but also demonstrate that these differences in size can also be reflected in changes in gene expression and craniofacial shape. This foundation can thus pave the way for future studies to expand upon these results by examining egg size-correlated variation at the individual level, and possibly conduct experiments until offspring sexual maturity to evaluate the extent of which maternal effects can persist over time, and whether offspring size-correlated craniofacial shapes can influence diet choice. This research contributes to the growing wealth of literature documenting mechanisms involved in the very early formation of different morphs (i.e. intraspecific diversity), an area of which is becoming increasingly important as biodiversity as a whole continues to decline.

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2 Paper I

Importance of egg size for phenotypic variation in sympatric and allopatric populations of polymorphic Arctic charr

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2.1 Abstract

Maternal effects have the potential to alter early developmental processes of offspring and contribute to adaptive diversification. However, to what extent intraspecific variation in maternal investment (i.e. egg size) may facilitate evolution of resource polymorphism is poorly understood. We studied an anadromous population and six resource morphs of Arctic charr in Iceland that range from an anadromous (and putatively ancestral) population to weakly and strongly divergent intra-lacustrine morphs. We characterised variation in egg size and tested whether egg size influenced offspring phenotype at early life stages (i.e. timing of and size at hatching and first feeding). We predicted that mean egg size should differ across morphs due to different environmental conditions, and that variation in egg size would be reduced in more diverged and derived morphs if selection has facilitated canalisation. We found that the anadromous (river Fljótaá) and the weakly diverged morph pair (from lake Vatnshlíðarvatn) had relatively small eggs, whereas the most divergent morphs (from lakes Þingvallavatn and Galtaból) had large eggs. We further found that egg size variation declined along the gradient of divergence, suggesting canalisation of egg size. Morphs/populations differed in timing of hatching and first feeding, as well as in offspring size at these stages. Egg size had no significant effect on developmental rates or size at hatching. However, larger eggs generally gave rise to larger offspring, but the magnitude of this effect varied across the morphs. These findings suggest that the timing and size at early life-history stages differs among resource morphs, in part explained by variation in egg size. We propose that variation in egg size may provide initial variation in developmental processes of offspring that consequently facilitates the evolution of resource polymorphism.

2.2 Introduction

Transgenerational plasticity, whereby the effects of the parental phenotype or environment influence offspring phenotype and performance (Mousseau & Fox 1998; Agrawal et al. 1999; Wolf & Wade, 2009), are an important means of adaptation to variable environmental conditions. Maternal effects commonly arise from egg size and content (Bernardo 1996), which can influence early development and how offspring development is adjusted under the constraints of the egg (West-Eberhard, 2003). Differences in maternal deposition of cytoplasmic factors (e.g. mRNAs, miRNAs, hormones, yolk) in different sized eggs typically influences offspring size (Chambers & Leggett, 1996; Heath & Blouw, 1998; Leblanc et al., 2016), but can also influence offspring plasticity. Evidence from both sea urchins (genus *Strongylocentrotus*: McAlister, 2007) and brook trout (*Salvelinus fontinalis*: Penney et al., 2018) suggest, for instance, that embryos originating from larger eggs are more developmentally plastic. Egg size can therefore affect phenotypic variation of offspring through developmental mechanisms, increasing intraspecific diversity from early life-stages (Allen et al., 2008) through to maturity (Hsu et al., 2016).

Both theoretical (Smith & Fretwell, 1974) and empirical (Einum & Fleming, 2000) evidence suggest a trade-off between offspring size and fecundity, whereby larger but fewer eggs may be favoured in poor growth environments, and smaller but more numerous eggs may be favoured in unfavourable environmental conditions (Hutchings, 1997; Einum et al., 2004; Räsänen & Kruuk, 2007). For example, in migratory Chinook salmon (*Oncorhynchus tshawytscha*), the cost of migration resulted in a decrease in egg size due to the physiological constraints of the female (Kinnison et al., 2001). Maternal per propagule investment can, however, vary strongly both within and among females even in the same environment since egg size variability has been found to reflect environmental unpredictability (Koops et al., 2003), as well as among populations inhabiting contrasting environments (e.g. Roff 1992; DeJong et al. 1995; Bernardo 1996).

Maternal effects can facilitate adaptive evolution and mediate divergence between populations (Räsänen & Kruuk, 2007), as seen in *Drosophila* (Eizadshenass & Singh, 2015), fish (Kinnison et al., 2001; Cogliati et al., 2018; Self et al., 2018; Thorn & Morbey, 2018), amphibians (Räsänen et al., 2003; Pfennig & Martin, 2009; Hangartner et al., 2012) and birds (Badyaev, 2002). For example, offspring of Chinook salmon (*Oncorhyncus tshawytscha*) originating from smaller eggs had deeper bodies than those originating from larger eggs (Cogliati et al., 2018). Likewise, in Arctic charr (*Salvelinus alpinus*), egg size is highly variable among and within females (Wallace & Aasjord, 1984; Baroudy & Elliott, 1994; Leblanc et al., 2016) and can influence feeding behaviour of offspring (Benhaïm et al., 2003; Leblanc et al., 2011). Specifically, larger eggs resulted in larger offspring that fed mainly from the surface, whilst smaller eggs gave rise to smaller offspring that moved less and spent more time on, or near to, the bottom (Benhaïm et al., 2003; Leblanc et al., 2011, 2016).

Variation in egg size and, subsequently, offspring size can provide a flexible source of phenotypic variation that has the potential to drive individual specialisation on alternative habitats or food resources, especially when competition is high (Bolnick et al., 2003; Cucherousset et al., 2011). Differences in size and developmental timing among individuals originating from different sized eggs could thus contribute to the initial resource-related diversification of morphs (i.e. resource polymorphism; Skúlason & Smith,

1995) if these phenotypic changes affect embryonic fitness by, for example, reducing resource competition at early life-stages. Individuals at different life history stages can also vary in their sensitivity to maternal effects, whereby maternal effects on offspring size often dissipate over the course of embryonic development (Heath et al., 1999; Lindholm et al., 2006). They can, however, in some cases persist to maturity (Sinervo & Doughty, 1996; Stelzer, 2002), such as seen for hatchery-reared Arctic charr where individuals that were small early in life were also amongst the smallest 2.5 years later (Bjorklund et al., 2003). Although relationships between egg size and offspring phenotype are becoming better known (Bernardo, 1996; Mousseau & Fox, 1998; Räsänen & Kruuk, 2007; Pfennig & Martin, 2009), we still lack information on the role of egg size in providing variation in developmental processes. Such information is of crucial importance in order to better understand the initial steps of divergence in the evolution of resource polymorphisms (Skúlason & Smith, 1995).

Resource polymorphism is common across numerous taxa, the processes of which has been considered as a diversifying force that may play a central role in population divergence and speciation (Smith & Skúlason, 1996; Levis et al., 2018). Resource polymorphism is particularly prominent in freshwater fishes (Seehausen & Wagner, 2014). In the northern hemisphere, the depauperate nature of post-glacial lakes has facilitated the evolution of resource polymorphism by providing colonising fishes ample opportunity to diversify and specialise (Snorrason & Skúlason, 2004; Svanbäck & Persson, 2004). One such species famed for resource polymorphism is the Arctic charr, in which sympatric morphs have evolved in multiple lakes (Adams et al., 1998; Gíslason et al., 1999; Skúlason et al., 1999; Jónsson & Skúlason, 2000; Alekseyev et al., 2002; Noakes, 2008; Gordeeva et al., 2010; Klemetsen, 2010; Kapralova et al., 2011).

In general, landlocked populations of Arctic charr vary in their degree of sympatric polymorphism with populations in some lakes showing low phenotypic and genetic divergence (e.g. lake Vatnshlíðarvatn; Jónsson & Skúlason, 2000), whilst in other lakes morphological divergence is strong and morphs are reproductively isolated (e.g. lake Galtaból; Gíslason et al., 1999). Anadromous Arctic charr can be considered as the ancestral 'stem' (Wund et al., 2008) for all landlocked populations of Arctic charr. The divergence from an initial colonising anadromous population can be accompanied by a reduction in phenotypic plasticity as a result of canalisation (Waddington, 1942; Crispo, 2007), as seen in resource polymorphic Arctic charr (Parsons et al., 2011).

The magnitude of variability in egg size and its potential for mediating or maintaining the evolution of resource polymorphisms in wild populations has been little studied (Pfennig & Martin, 2009). If egg size-mediated maternal effects provide the initial phenotypic variation that selection – through resource availability – can act upon, ancestral populations should harbour more variation in egg size and show clear effects of egg size on offspring phenotype. Moreover, morphs at early stages of divergence should be more similar to the ancestral population, whereas more diverged (and likely specialised) morphs should be less variable in egg size and offspring phenotype due to trait canalisation (Parsons et al., 2011). In this vein, we here characterise variation in egg size across a gradient of phenotypic and genetic divergence, from an anadromous (river Fljótaá) population to weakly and strongly divergent landlocked intralacustrine morphs of Arctic charr. In the weakly diverged sympatric morph pair (Vatnshlíðarvatn), the morphs differ mostly in the degree of specialisation to a benthic diet (i.e. more specialised vs more generalist) and spawning behaviour (Jónsson & Skúlason, 2000; Parsons et al., 2011). The most divergent sympatric pairs consist of strongly divergent benthic and pelagic morphs that rarely interbreed (e.g. Þingvallavatn and Galtaból morphs; Sandlund et al., 1992; Gíslason et al., 1999; Kapralova et al., 2011; Parsons et al., 2011).

Here we predict that i) egg size differs among Arctic charr populations/morphs due to selection favouring different optimal egg sizes in different environments (Skúlason et al., 1989b; Smalås et al., 2017), and that ii) egg size will be most variable in anadromous charr (putative ancestral population) but due to canalisation will be least variable in the most strongly diverged (specialised) freshwater morphs. Finally, if egg size is an important determinant of offspring phenotype at early life-stages, egg size should influence timing and size at key transitions (here: hatching and first feeding stage). In particular, we expect offspring from smaller eggs to reach first feeding stage earlier than their larger conspecifics, as smaller per propagule maternal investment should be compensated by earlier onset of feeding (Eiríksson et al., 1999; Leblanc et al., 2016; Beck et al., 2018).

2.3 Materials and methods

2.3.1 Study systems, crosses and rearing of embryos

An anadromous population and six morphs were sampled in this study (Fig. 2.1). 1) An anadromous population, reflecting a putative ancestral phenotype of purely freshwater Arctic charr and was caught in Fljótaá (FJ; a river in North Iceland). The females and males from this river were larger and had stronger red colouration than the landlocked charr morphs (personal observations). 2 and 3) Two morphs from lake Vatnshlíðarvatn (brown, VB, and silver, VS), that are weakly diverged (both phenotypically and genetically; Gíslason et al., 1999) and differ in both time and place of spawning were collected. This lake is physically simple, small and shallow (Jónsson & Skúlason, 2000). VB has a more specialised benthic diet, feeding primarily on *Eurycercus* sp., whereas the VS morph is more of a generalist benthic feeder feeding upon *Pisidium* sp., *Eurycercus* sp. (although less so compared to the brown morph), Chironominae and Tanypodinae (Jónsson & Skúlason, 2000; Franklin et al., 2018). The degree of morphological differentiation is small (but significant) between these two morphs, and no clear differences in genotype frequencies, although overall genetic differentiation did differ (Gíslason et al., 1999). This is likely due to their different spawning strategies with VS spawning in streams, whilst VB spawns within the lake. 4) A pelagic morph from lake Svínavatn (SV), a large, deep, lake harbouring three Arctic charr morphs: a pelagic planktivorous (studied here), piscivorous and a benthic morph. The pelagic morph differs from the other two morphs in shape as well as at one genotypic locus (Gíslason et al., 1999). Although diet is currently unknown, we suspect (based on it being a pelagic morph) that the diet is primarily planktivorous, and spawns in the shallows on the east and west side of lake Svínavatn. 5 and 6) A strongly divergent large benthic (TLB) and a pelagic planktivorous (TP) morph from Iceland's largest natural lake, Þingvallavatn, which is inhabited by four morphs (large benthic, small benthic, pelagic planktivorous and piscivorous). TLB and TP differ in morphology from very early in development (Skúlason et al., 1989a; Kapralova et al., 2015) reflecting their trophic specialisations. TLB feeds on zoobenthos in the littoral stony habitat, particularly the gastropod *Lymneaea*, whilst TP feeds primarily upon zooplankton in the pelagic habitat of the lake (Malmquist et al., 1992). Pronounced phenotypic differences exist between these two morphs (Sandlund et al., 1992), which are also accompanied by genetic differentiation, albeit weaker than phenotypic differences (Magnusson & Ferguson, 1987; Volpe & Ferguson, 1996; Kapralova et al., 2015). 7) A highly diverged (both phenotypically and genetically; Gíslason et al., 1999) benthic morph from lake Galtaból (GB). The lake is a small highland lake, which harbours two morphs: a piscivorous (a specialised stickleback predator) and a benthic morph that are reproductively isolated (Gíslason et al., 1999; Coyne & Orr, 2004). All populations/morphs were caught at the end of summer and in autumn of the years 2014-2016: FJ = 2015 and 2016; VB = 2014, 2015, VS = 2014, 2015; SV = 2015 and 2016; TLB = 2014, 2015; GB = 2014, 2015; TP = 2014:2016. Sampling was conducted in different years for logistical reasons. Those that spawned within lakes were collected using gill nets, and those that spawned within streams collected using electrofishing.

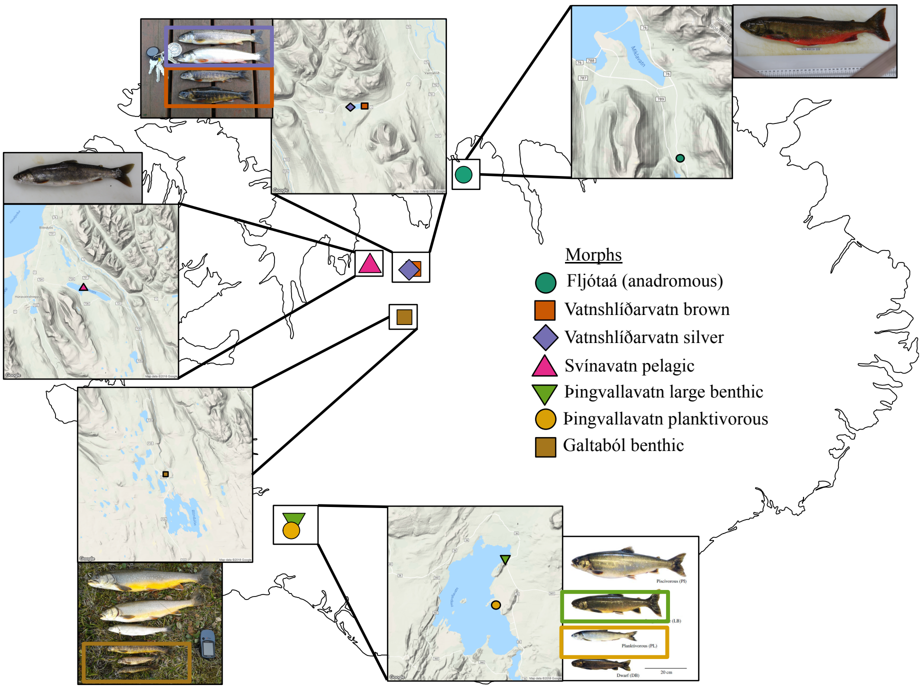

Figure 2.1 *Map of Iceland showing sampling locations for the anadromous population and six Arctic charr morphs sampled in this study. In cases where multiple morphs co-exist, only those used in this study are highlighted in a coloured frame. All morphs spawn within lakes except for Vatnshlíðarvatn silver (which migrates to streams to spawn) and the anadromous Fljótaá. Lake locations are zoomed in at the same scale (x10) for each lake. Image of morphs from lake þingvallavatn were modified from Johnston et al. (2004).*

Maternal phenotype

Mature females (N = 14 [VS] to 24 [TP], see Table 2.1 and Table S2.1 for more detailed information on sample sizes) were mated to a single male in the field. In most cases (see Table S2.1) the same male was used to fertilise multiple females to standardise any genetic background that is not due to the mother. Fertilised eggs were allowed to water-harden before transport to Hólar University College's aquaculture facilities in Verið, Sauðárkrókur. Females were sacrificed and weighed in the field to the nearest 0.1g, before being brought back to the lab and measured for fork length (FL; to the nearest 0.1cm), the left-side of whole individuals were photographed with a scale (Canon EOS 650D), and a caudal fin clip stored in 96% ethanol to allow later genetic analyses.

To estimate age, sagital otoliths were removed from each individual and cleaned in alcohol from any bloodstains and tissue. After drying overnight, otoliths were stored at room temperature until reading. Scaled photographs of otolith pairs were taken under a stereomicroscope (Leica M165 C), so that age of individuals could be inferred from counting annuli at the surface of each otolith. Depending on the thickness of the otoliths – which varied across populations/morphs – otoliths were immersed in 96% ethanol for 8-20 hours in order to optimize the readability of the annuli at both the core and the edges. Otoliths immersed in ethanol were photographed against a dark background under reflected light using a LEICA mounted camera. Otoliths were read twice under the microscope and twice from enhanced images. In cases of any discrepancies in age estimation, otoliths were revisited and a final age estimate was obtained following (Tsinganis, 2016). Ageing of females were conducted by M. Tsinganis and A. Reilent, the methods of which can be found in more detail in Tsinganis (2016).

Rearing of embryos

Embryos were reared in family groups in cages situated in an incubator system using standard procedures (Beck et al., 2018). The cages (6.5cm x 6.5cm x 5cm) had 2cm x 2cm holes at the front and rear. The holes and the bottom of the cage were covered with mesh (1mm mesh size) to ensure good oxygenation. Cages were placed in a shelf incubator system (MariSource 8-tray Vertical Incubator) with a constant flow of 95% recycled water. For most females, a single cage was used (i.e. one layer of eggs), but when eggs were numerous they were split into different cages to allow efficient oxygenation. Embryos were reared in darkness to mimic natural conditions, and at constant temperature (4.25ºC ± 0.48 SD). Temperature was monitored using HOBO loggers (to the nearest 0.01°C) four times daily. Dead individuals (opaque eggs) were manually removed every two days to prevent fungal growth. An accumulative temperature estimate (degree days, DD; Pruess, 1983) was used to determine the approximate time by which certain developmental stages would occur (see below).

Table 2.1 *Female and offspring measurements in the anadromous population and six morphs of Arctic charr (Salvelinus alpinus) found in allopatry and sympatry throughout Iceland. The seven populations/morphs are FJ, Fljótaá (ancestral anadromous population); VS, Vatnshlíðarvatn silver; VB, Vatnshlíðarvatn brown; SV, Svínavatn; TLB, Þingvallavatn large benthic; TP, Þingvallavatn planktivorous; and GB, Galtaból benthic. Each female was sampled for mean egg size (diameter, mm; ± standard deviation) a few hours after fertilisation, and for embryo size (SL, standard length, mm) at hatching (H) and first feeding (FF).*

	Female			Egg size						Hatching stage					First feeding				
Morph	**N**	**Mean age**	**Mean FL**	**N ♀**	**Mean size**	**CV (%)**	**N off.**	**PF DD**	**E DD**	**N ♀**	**Mean size**	**CV (%)**	**N off.**	**DD**	**N ♀**	**Mean size**	**CV (%)**	**N off.**	**DD**
FJ	15	5 ± 1.3	37.7 ± 3.87	15	4.5 ± 0.20	6.1	810	10 ± 0.49	201 ± 2.89	15	14.8 ± 1.09	7.4	429	425 ± 23.10	15	20.2 ± 0.99	4.9	359	653 ± 29.42
VS	14	6 ± 0.8	24.4 ± 1.87	14	4.5 ± 0.23	5.9	476	11 ± 1.04	220 ± 2.49	14	14.3 ± 1.08	7.6	221	419 ± 21.30	14	18.7 ± 0.99	5.3	273	637 ± 8.60
VB	17	6 ± 1.5	18.0 ± 3.84	16	4.4 ± 0.49	12.1	640	22 ± 7.39	229 ± 7.89	15	14.6 ± 1.27	8.7	245	452 ± 21.97	15	17.8 ± 1.85	10.4	283	678 ± 29.06
SV	17	5 ± 1.3	25.6 ± 1.59	8	5.3 ± 0.22	5.5	367	10 ± 0.49	203 ± 1.31	4	15.4 ± 1.15	7.5	63	417 ± 25.55	4	22.8 ± 1.40	6.2	38	725 ± 29.06
TLB	17	9 ± 2.2	36.0 ± 5.27	15	5.1 ± 0.19	5.1	788	14 ± 1.88	212 ± 6.59	14	15.4 ± 1.45	9.4	174	443 ± 8.33	14	20.5 ± 1.11	5.4	280	666 ± 23.37
TP	24	6 ± 1.0	20.7 ± 1.28	22	5.0 ± 0.16	5.6	483	10 ± 4.20	227 ± 24.33	24	16.7 ± 0.96	5.7	277	448 ± 13.31	23	21.1 ± 1.16	5.5	313	652 ± 21.71
GB	15	6 ± 0.9	21.8 ± 1.88	14	5.1 ± 0.18	4.1	334	11 ± 0.53	229 ± 1.83	4	14.8 ± 0.73	5.0	74	441 ± 11.70	12	19.8 ± 1.64	8.3	124	619 ± 0.64

CV, coefficient of variation. Age, age in years. H, hatching. FF, first feeding. SL, standard length (mm). FL, fork length (cm). N, sample size. ♀, female. Note that sample sizes of females were too small to use developmental timings of SV and GB for statistical analyses. For number of offspring used per female and developmental stage, see Table S2.1. Total number of offspring across all developmental stages were: FJ = 1598, VS = 970, VB = 1168, SV = 468, TLB = 1242, TP = 1073 and GB = 532.

2.3.2 Sample collection

To characterise variation in size and developmental time, we sampled the anadromous population and six morphs for mean offspring size (embryonic diameter or standard length, SL) per female at four developmental stages and relevant degree days (DD). At 1) post-fertilisation (PF, DD = 13 ± 4.39 SD), 2) eye stage (E; DD = 217 ± 12.07 SD), when eye lenses are formed and retinas pigmented, 3) hatching (H, DD = 435 ± 14.36 SD), when individuals have hatched out of the confines of the egg but still rely on nutrition from the yolk sac, and 4) first feeding (FF, DD = 661 ± 33.90 SD), when individuals initiate feeding (Ballard, 1973), either by feeding off the bottom or by swimming up to take food from the surface or water column. Sampling at each of the four stages occurred when approximately 50% of individuals within a given family had reached that particular stage. The number of DDs at this time point was used as a measure of developmental time for each clutch.

To obtain an accurate coverage of offspring size range within each female, we visually selected embryos at the PF and E stages based on their size (small, medium and large), according to validated methods conducted in previous studies (Benhaïm et al., 2003; Leblanc et al., 2011; Table 2.1). At H and FF, individuals were chosen randomly since size selection was considerably more difficult. An absolute minimum of three individuals per female/developmental stage was collected (see Table S2.1). All individuals (eggs, or left-side of H and FF embryos) were digitally photographed (Canon EOS 650D, 100mm macro lens) with a scale, and eggs measured four times each to obtain average diameter (PF and E), and just once for measurement of SL (for H and FF stages; Leblanc et al., 2016) to the nearest 0.01mm, using the program Fiji (Schindelin et al., 2012). Only females with >15 eggs for measures of egg size (combined PF and E) were kept for further analyses to ensure accurate estimates of mean and variation in egg size. Differences in the number of females sampled across developmental stages arose due to offspring mortality or sampling error. In particular, GB and SV had small sample sizes at H (female N = 4) and SV also at FF (female N = 4). In all other cases, a minimum of 11 females were sampled at each developmental stage (see Table 2.1).

2.3.3 Statistical analyses

All statistical analyses were conducted on female means in R (version 3.3.2, R Core Team, 2016) and all model residuals investigated using QQ plots and histograms to test for normality and heteroscedacity.

To test for differences in female phenotype among morphs, we ran factorial linear models to test whether female FL differed with female age and morph (unless otherwise specified, for brevity within the Analyses and Results we will refer to the term ‘morph’ as also encompassing the anadromous population) as fixed factors, and the interaction between the two. The main response variables for offspring related traits were female means of egg size, coefficient of variation of egg size ($CV_{eggSize}$), DD and size at hatching as well as DD and size at first feeding.

Egg size and $CV_{egg\ size}$ within and among morphs

In fishes, egg size is typically positively related to female length (van den Berghe & Gross, 1989) and can also be correlated with female age (Lasne et al., 2018). For analyses of egg size, we only report results using female FL as a covariate in the models since this was preferred (over models with age) in models both on egg size and $CV_{eggSize}$ based on initial model fit using Akaike Information Criterion (AIC).

We analysed both absolute egg size and relative egg size (accounting for female size by including female fork length as a covariate). We used absolute egg size as it is strongly correlated with per propagule maternal investment in yolk and most directly linked to offspring phenotype and fitness. Previous studies further show that absolute egg size affects offspring morphology in salmonids (Cogliati et al., 2018). We also used relative egg size as it is indicative of propagule maternal investment accounting for female phenotype. Both absolute and relative egg size were compared across the morphs using linear models with morph as a fixed factor, and female FL and its interaction with morph as a covariate (in analyses of relative egg size). Additionally, coefficient of variation in egg size ($CV_{eggSize}$, calculated by dividing the standard deviation of measures of egg size by mean egg size per morph) was analysed among the morphs using linear models on both absolute and relative egg sizes with morph as a fixed effect and female FL (where appropriate) as covariate.

When there were significant morph effects, planned contrasts using *post-hoc* Tukey adjusted means were conducted using pairwise comparisons of the ancestral FJ population against all other morphs, as well as pairwise comparisons between sympatric morphs within lakes. We specifically predicted that the magnitude of pairwise differences should be weakest between FJ and the VS or VB, and relatively stronger between FJ and TLB, TP or GB, as well as being weaker between the sympatric VS and VB than between the sympatric TLB and TP morphs. Pairwise comparisons of slopes were conducted using the *emtrends* function from the estimated marginal means (EMM; *emmeans*) package (Lenth, 2017). The *pairs* function was then used to test for slope deviations from zero. Comparisons of trait means were conducted using least square means from the *lsmeans* package (Lenth, 2016).

Egg size effects on offspring phenotype

To test the effect of female mean egg size on offspring phenotype (developmental time and size at hatching and first feeding), we used separate factorial linear models within each developmental stage (H and then FF) on female means. These models included morph as a fixed effect. To test for slope heterogeneity between egg size and offspring phenotype between morphs, a morph x egg size interaction was included in each model. If the interaction was not significant, it was excluded from the model (only results of final model are presented). Based on sampling error, the date when 50% of offspring within a given cage had hatched or reached the first feeding stage was only accurate for a subset on females which were then averaged to estimate the female mean DDs at these stages for all females from FJ and a subset of TLB (females 116, 119, 124, 128) and TP (females 240, 250:253) females. All other estimates of DD days came from individual rearing data (N = >7; Table S2.2), which were subsequently averaged per female for statistical analyses for TLB, TP, VB and VS. It must be noted that TLB an TP dates overlapped between the average date at which offspring from each female starts to hatch or feed, and the date at which offspring from cages started to hatch or feed. Only SV and GB were not included in analyses of developmental timings since sample sizes were too small.

2.4 Results

2.4.1 Female phenotype

Female FL differed among morphs ($F_{6, 207}$ = 224.9, *P* <0.0001), age ($F_{1, 207}$ = 35.03, *P* <0.0001) as well as the interaction of the two ($F_{6, 207}$ = 2.63, *P* = 0.018; Fig. 2.2, 3). Females of the FJ and TLB morphs were the largest (37.7cm, SD ± 3.87 and 36.0cm, SD ± 5.27, respectively). Females from FJ were larger than females of all other morphs (Pairwise Tukey tests: all P < 0.0001, Fig. 2.2) apart from TLB (*P* = 0.999). In contrast, FJ females were on average younger (mean ± SD: 5 ± 1.3 years; Fig. 2.2b) while TLB females were older (mean ± SD: 9 ± 2.2 years, Fig. 2.2b) than females from other morphs (Fig. 2.2b). For the Vatnshlíðarvatn morphs, VB females were smaller than the VS, despite both being of similar age (Fig. 2.2). For þingvallavatn fish, the TLB morph was both substantially larger and older than the TP morph (Fig. 2.2a,b). Despite the significant morph x age interaction, there were no significant differences among the slopes of age and female FL among any of the morphs in pairwise comparisons (P = >0.05; Fig. 2.3). However, slopes in VB and TLB differed significantly from zero (*t* = 4.77, *P* = <0.0001 and *t* = 4.77, *P* = <0.0001; Fig. 2.3).

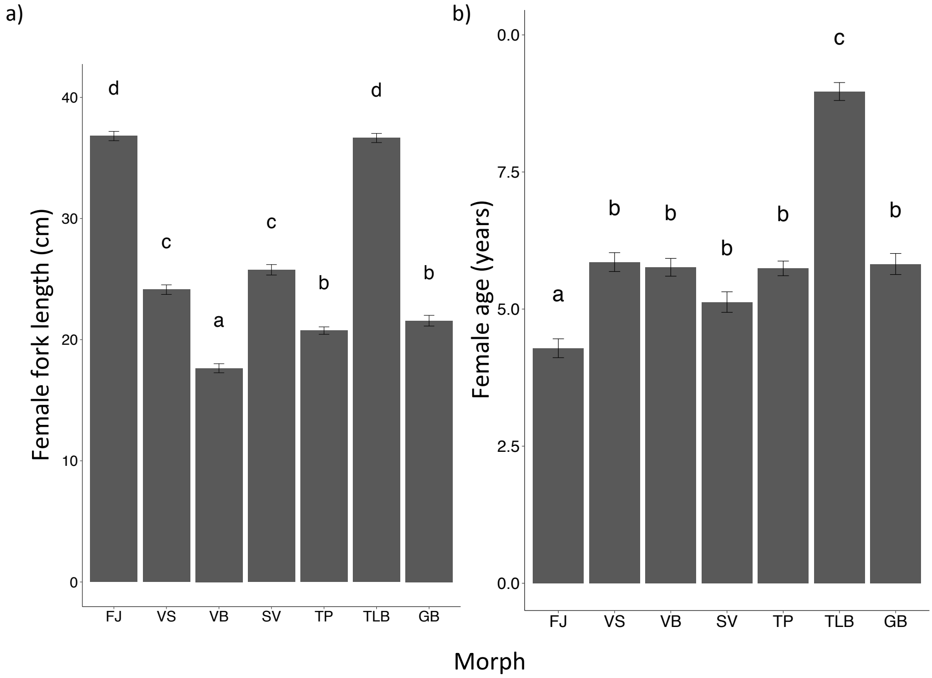

Figure 2.2 *Adjusted means from a linear model examining differences in a) female fork length (cm) and b) female age (years) in the anadromous population and six morphs of Arctic charr: FJ, Fljótaá; VS, Vatnshlíðarvatn silver; VB, Vatnshlíðarvatn brown; SV, Svínavatn; TP, Þingvallavatn planktivorous; TLB, Þingvallavatn large benthic; GB, Galtaból benthic. Different letters correspond to significant pairwise differences between morphs (at P <0.05) from post-hoc Tukey tests.*

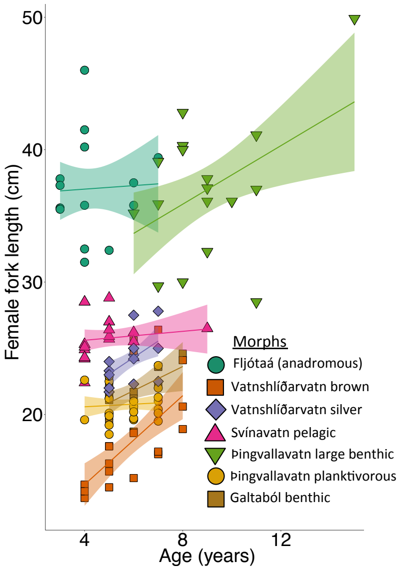

Figure 2.3 *Relationship between female fork length (FL) and age in in the anadromous population and six Arctic charr morphs with 95% confidence level intervals for predictions from a linear model. Despite a significant morph x age interaction (see Results text), pairwise comparisons of slopes revealed no differences between morphs. The data points represent female means.*

2.4.2 Egg size differences within and among morphs

Egg size differed among the morphs ($F_{6,\ 216}$ = 56.56, *P* <0.0001; Fig. 2.4) and remained different after accounting for female FL ($F_{1,\ 209}$ = 35.59, *P* <0.0001; Table 2.2). There was also an interaction between female FL and morph ($F_{6,\ 209}$ = 11.35, *P* <0.0001). The morph with the smallest egg size was VB (4.44 mm, SD ± 0.49), whilst the largest egg size was in SV (5.33 mm, SD ± 0.22). *Post-hoc* pairwise comparisons of least square means for absolute egg size found that 1) FJ, VS and VB which had the smallest eggs did not differ from each other, whereas 2) TLB, TP and GB, which had the largest eggs, did not differ from each other (*P* values for significant contrasts are shown in Table S2.3). SV had larger eggs than all morphs (Fig. 2.4a). After accounting for female FL (Fig. 2.4b), FJ and VS had smaller eggs for a given female size than the other morphs, whereas none of the other morphs differed from each other in relative egg size (Fig. 2.4b).

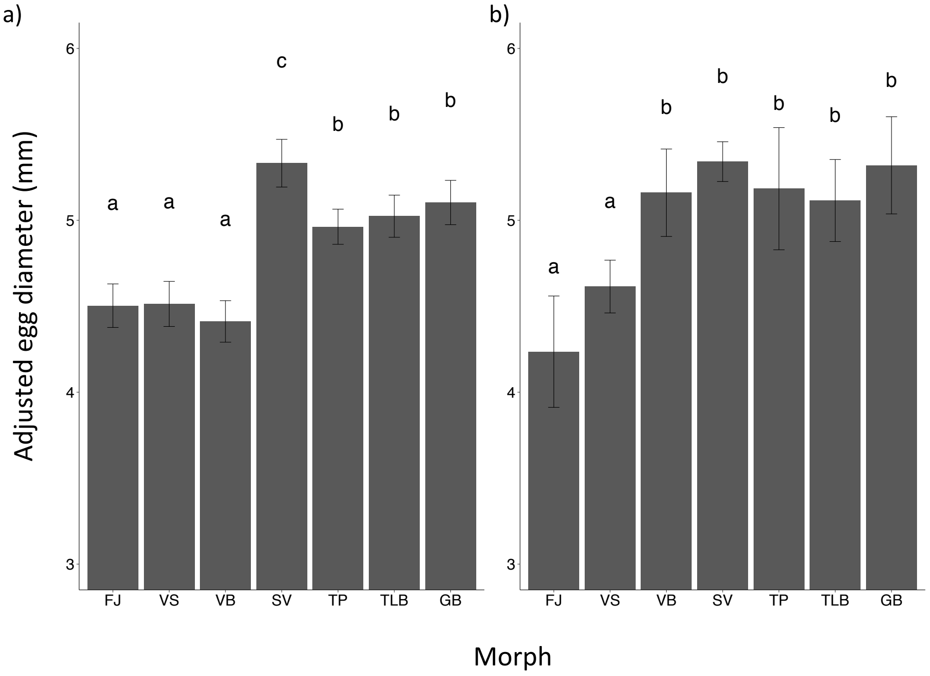

Figure 2.4 *Adjusted means from a linear model testing differences in a) absolute egg size and b) relative egg size (after accounting for female size), in the anadromous population and six morphs of Arctic charr (FJ, Fljótaá; VS, Vatnshlíðarvatn silver; VB, Vatnshlíðarvatn brown; SV, Svínavatn; TP, Þingvallavatn planktivorous; TLB, Þingvallavatn large benthic and GB, Galtaból benthic.*

Slopes of egg size against female FL show most morphs to increase in egg size with female size except TLB (Fig. 2.5). The strongest relationship between female FL and egg size was found for VB (slope*:* $t = 8.68$, $P < 0.0001$). Significant differences between slopes were found between FJ and VB ($t = -4.56$, $P < 0.001$) as well as TLB and VB ($t = -7.84$, $P < 0.001$; Table S2.3). (N.B. as female FL and morph are partially confounded [no overlap between FJ or TLB and the remaining morphs] we can not clearly delineate whether the differences between morphs in absolute and relative egg size are due to female size or morph identity).

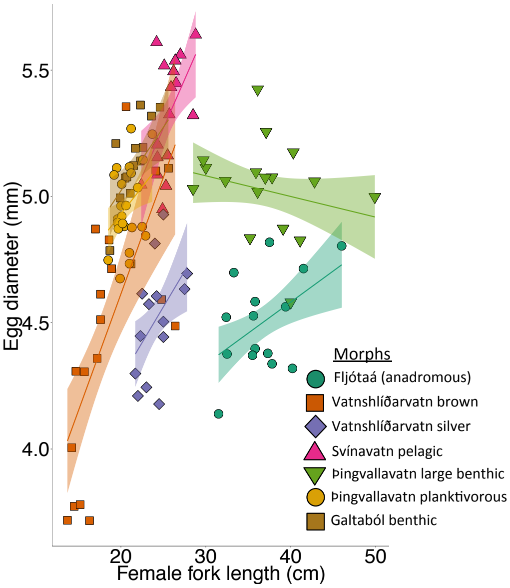

Figure 2.5 *Relationships between female fork length (FL; cm) and egg diameter (mm) in the anadromous population and six Arctic charr morphs, with 95% confidence level intervals for predictions from a linear model testing female FL x morph. Slopes were significantly different from zero for Vatnshlíðarvatn brown (P <0.0001). Slopes were significantly different between the FJ and VB morph (P <0.001) as well as VB and TLB (P <0.0001). The data points represent female means.*

Table 2.2 *Results of linear models a) female mean egg size and coefficient of variation (CV) of egg size and b) relative female mean egg size and coefficient of variation (CV) of relative egg size (accounting for female size) as well as c) offspring phenotype in the anadromous population and six Arctic charr morphs.*

Response variable	N	Factor	Sum Sq	DF	F	*P*
a) Absolute egg size						
Mean egg size	223	**Morph**	**22.26**	**6**	**56.56**	**<0.0001**
		Residuals	14.17	216		
Egg size variation (CV)	223	**Morph**	**0.02**	**6**	**13.13**	**<0.0001**
		Residuals	0.06	216		
b) Relative egg size						
Egg size	223	**Female FL**	**1.61**	**1**	**35.59**	**<0.0001**
		Morph	**23.40**	**6**	**86.07**	**<0.0001**
		Female FL * Morph	**3.09**	**6**	**11.35**	**<0.0001**
		Residuals	9.47	209		
Egg size variation (CV)	223	Female FL	0.00	1	1.13	0.29
		Morph	**0.02**	**6**	**13.35**	**<0.0001**
		Female FL * Morph	**0.01**	**6**	**5.29**	**<0.0001**
		Residuals	0.05	209		
c) Offspring traits						
Timing of hatching	45	**Morph**	14609.60	4	25.61	**<0.0001**
		Residuals	5705.90	40		
Timing of first feeding	45	**Morph**	5689.60	4	3.45	**0.016**
		Residuals	16494.50	40		
Timing of hatching (after accounting for egg size)	45	Egg size	8.00	1	0.05	0.816
		Morph	**14337.10**	**4**	**24.53**	**<0.0001**
		Residuals	5697.80	39		
Timing of first feeding (after accounting for egg size)	45	Egg size	1089.30	1	2.76	0.105
		Morph	**5150.60**	**4**	**3.26**	**0.021**

		Residuals	15405.20	39		
Size at hatching	92	Egg size	2.06	1	2.86	0.094
		Morph	**41.80**	**6**	**9.68**	**<0.0001**
		Residuals	60.43	84		
Size at first feeding	97	**Egg size**	**25.56**	**1**	**47.35**	**<0.0001**
		Morph	**75.23**	**6**	**23.23**	**<0.0001**
		Egg size * Morph	**9.50**	**6**	**2.93**	**0.012**
		Residuals	44.80	83		

N, number of females across all morphs, Df, degrees of freedom. F, F-statistic. P, P-value based on F statistic. Sum Sq, Sum of squares.

2.4.3 Variation in egg size within and among morphs

Female means of coefficient of variation in egg size ($CV_{eggSize}$) differed among the morphs both before and after accounting for female FL (Fig. 2.6; Table 2.2). Before accounting for female FL, VB had the highest coefficient of variation in egg size ($CV_{eggSize}$ = 12.1%), whilst GB had the lowest ($CV_{eggSize}$ = 4.1%, Table 2.1; Fig. 2.8). *Post-hoc* pairwise comparisons of $CV_{eggSize}$ prior to accounting for female FL found that $CV_{eggSize}$ was lower in FJ than in VB and higher in FJ than GB morphs (Fig. 2.8a), whereas other morphs did not differ from FJ. $CV_{eggSize}$ was higher in VB compared to the sympatric VS, but did not differ between TLB and its sympatric TP morph.

After accounting for female FL, FJ had the relatively highest variation in egg size (adjusted means of $CV_{eggSize}$ = 6.9%; results not shown) and TP the relatively lowest (0.9%), Fig. 2.8b. Female size-adjusted $CV_{eggSize}$ did not differ between FJ and VS and VB morphs (adjusted means of $CV_{eggSize}$ for VS = 4.1% and VB = 5.9%; Fig. 2.8), but was different between FJ and the remaining landlocked morphs (SV, TLB, TP, GB). The sympatric TLB and TP morphs did not differ significantly although TP had very low variation in egg size (CV = 0.9%; Fig. 2.8). After accounting for female FL, there is a general decrease in variation in egg size from the most ancestral anadromous FJ to the most diverged landlocked GB morph (Fig. 2.8).

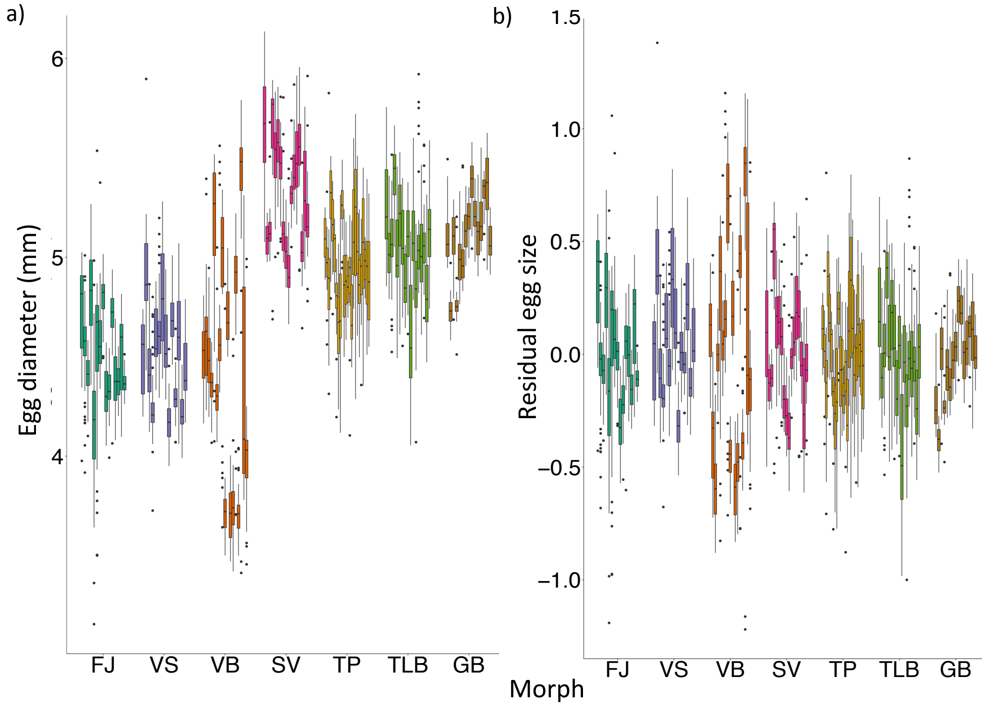

Figure 2.6 *Egg size variation per each individual female in the anadromous population and six morphs of Arctic charr for a) absolute egg size and b) residuals of egg size (i.e. after accounting for female fork length). Median and interquartile ranges are shown for each single female. Colours indicate the different morphs: FJ, Fljótaá; VB, Vatnshlíðarvatn brown; VS, Vatnshlíðarvatn silver; SV, Svínavatn; TLB, Þingvallavatn large benthic; TP, Þingvallavatn planktivorous; and GB, Galtaból benthic.*

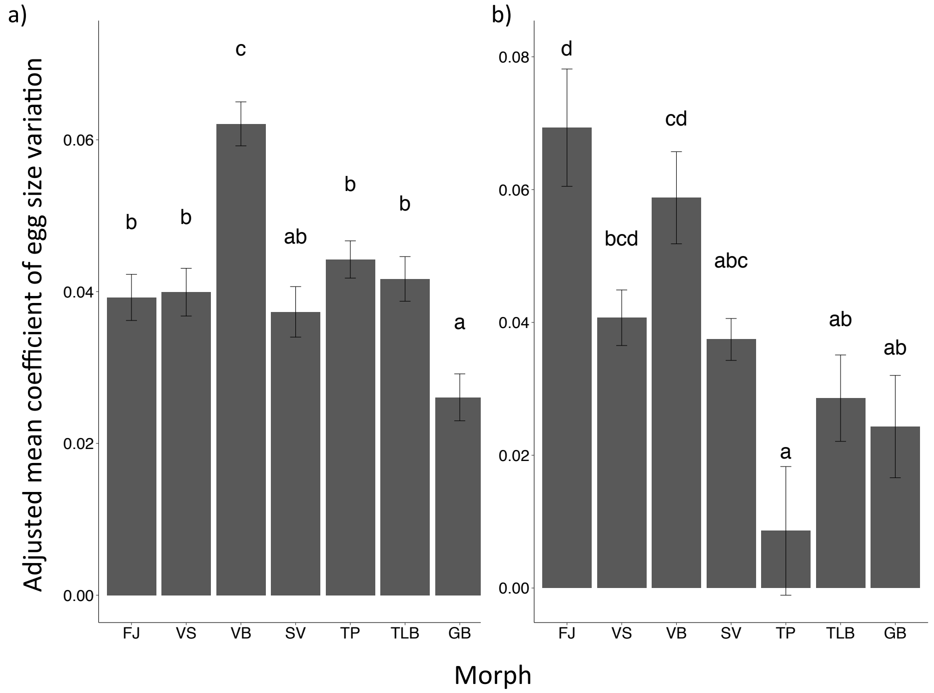

Figure 2.7 *Adjusted means from a linear model testing differences in a) absolute and b) relative (after accounting for female fork length (FL) coefficient of variation in egg size (*$CV_{eggSize}$*) in the anadromous population and six morphs of Arctic charr: FJ, Fljótaá; VS, Vatnshlíðarvatn silver; VB, Vatnshlíðarvatn brown; SV, Svínavatn; TP, Þingvallavatn planktivorous; TLB, Þingvallavatn large benthic; and GB, Galtaból benthic. The letters indicate pairwise significance between morphs at P <0.05 (shared letter = not significantly different).*

Slopes between FL and $CV_{eggSize}$ differed from zero in FJ (t = -3.60, P = 0.003) and TP (t = *-3.788, P* =0.001). These female size-adjusted $CV_{eggSize}$ slopes did not differ between FJ and VS or VB but did differ between FJ and SV, TLB, TP and GB (Fig. 2.8; Table S2.3). There was no difference in the relative $CV_{eggSize}$ slopes between VS and VB or between TLB and TP. As above note that female FL and morph are likely confounded, therefore rendering it difficult to distinguish whether the $CV_{eggSize}$ is due to female FL or morph identity.

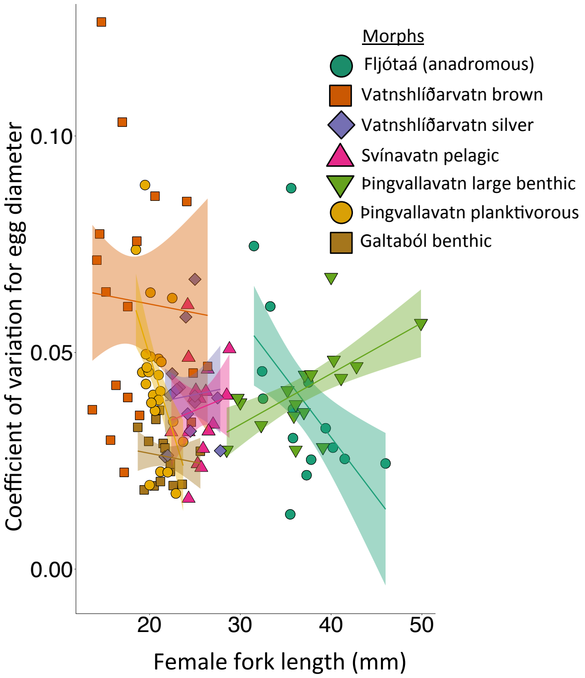

Figure 2.8 *Relationship between female mean variation in egg size ($CV_{eggSize}$) and female fork length (FL) in the anadromous population and six Arctic charr morphs, with 95% confidence level intervals for predictions from the linear model (Morph x Female FL). Pairwise comparisons indicated no significant differences between the anadromous Fljótaá population and the two lake Vatnshlíðarvatn morphs (VS and VB) but FJ slopes were significantly different from all other morphs (all P <0.05; Table S2.3). The data points represent female means.*

2.4.4 Egg size and offspring phenotype

Offspring size at H and FF stage as well as developmental time (DD) until H and FF stages differed among the morphs (Table 2.2). Both before and after adjusting for egg size, the anadromous FJ hatched substantially earlier than all landlocked morphs (all pairwise *P* <0.001; Fig. 2.9; Table S2.3). The sympatric VS and VB differed in time to hatching with VB developing slower (i.e. hatching later) than VS before (*t* = -29.55, *P* = <0.001) and after accounting for egg size (*t* = 29.37, *P* <0.001; Table S2.3, Fig. 2.9a), but there was no difference between TP and TLB. In contrast to timing until H, FJ reached FF stage later than the VS morph, which developed fastest to FF both before (*t* = 43.68, *P* = 0.008) and after accounting for egg size (*t* = 42.16, *P* = 0.01), Fig. 2.9. The other morphs did not differ in timing until FF.

The morphs differed in size at H, with TP being larger than those from other morphs (*P* <0.05), with the exception of SV (Fig. 2.10a; Table S2.3). Morph differences in offspring size were more pronounced at first feeding, with offspring from sympatric morphs being more similar in size than allopatric morphs (Fig. 2.10b; Table S2.3, pairwise comparisons: <0.05). After accounting for egg size, offspring from TP remain larger at hatching in comparison to all other morphs (*P* <0.01; Table S2.3) except for SV (Fig. 2.11a; Table S2.3; pairwise comparisons: <0.01), but there was no relationship between size at H and egg size (*P* 0.094).

Egg size had no effect on timing of H or FF, or on size at H (Table 2.2, Fig. 2.11). However, egg size was related to size at FF and, indicated by a significant morph x egg size interaction (Table 2.2), the effects of egg size on size at FF differed among morphs (Fig. 2.11b). These effects arose mostly from offspring from larger eggs feeding at a larger size in GB compared to VB and VS (slopes: *P* <0.05 for both; Table S2.3).

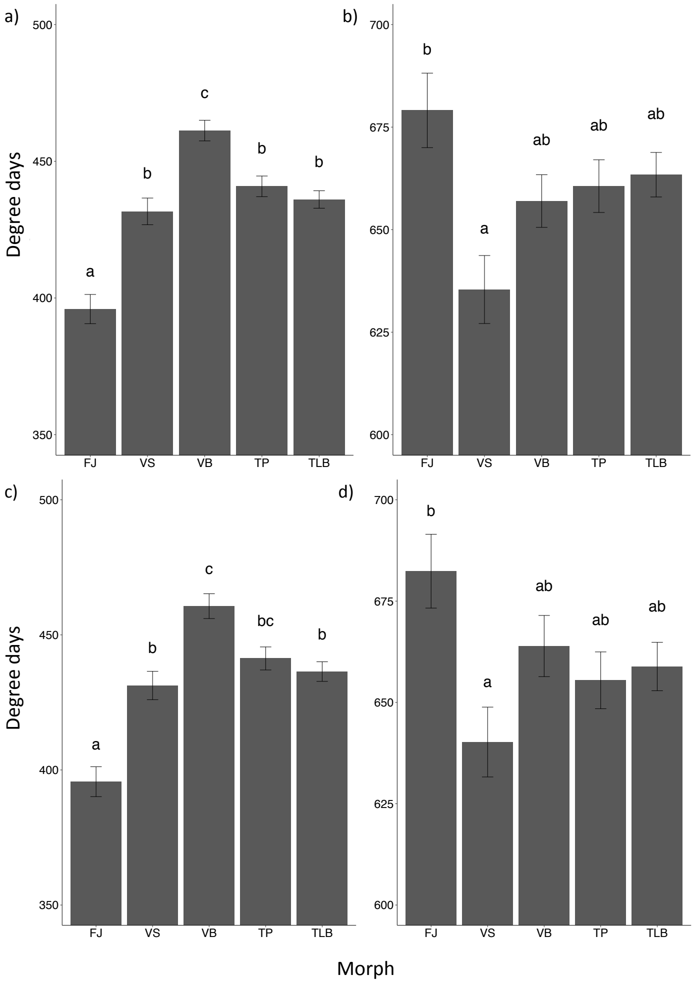

***Figure 2.9** Adjusted means of degree days (DD) at a) hatching (H), b) first feeding (FF) stage, c) H after accounting for egg size, and d) FF after accounting for egg size, from linear models (Table 2.2) in the anadromous population and four morphs of Arctic charr (FJ, Fljótaá; VB, Vatnshlíðarvatn brown; VS, Vatnshlíðarvatn silver; TLB, Þingvallavatn*

large benthic; and TP, Þingvallavatn planktivorous; Table 2.2). *Significance (P < 0.05) in pairwise differences among morphs are indicated by differences in letters.*

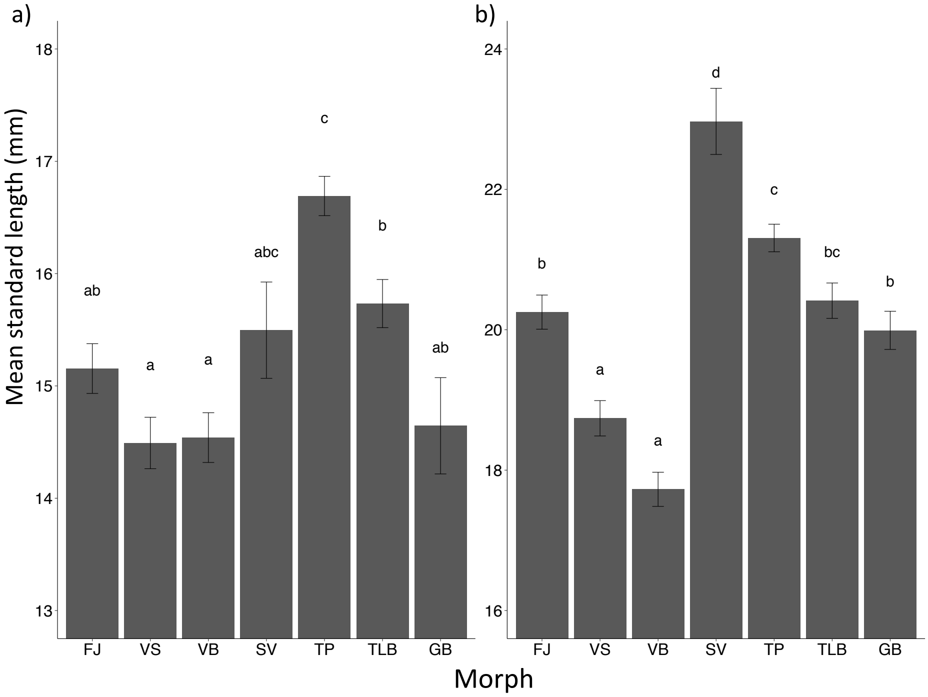

Figure 2.10 *Adjusted means of standard length (mm) at a) hatching (H), and b) first feeding (FF) for the anadromous population and six morphs of Arctic charr (FJ, Fljótaá; VS, Vatnshlíðarvatn silver; SV, Svínavatn; VB, Vatnshlíðarvatn brown; TP, Þingvallavatn planktivorous; TLB, Þingvallavatn large benthic; and GB, Galtaból benthic) with associated standard errors. Data is based mean offspring size per female.*

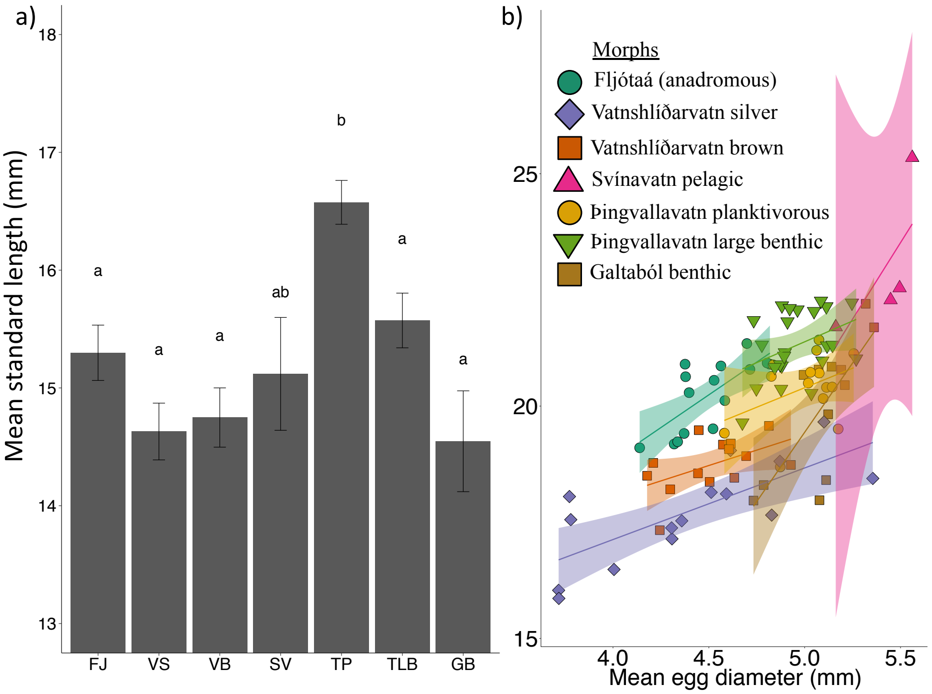

***Figure 2.11** Pairwise comparisons of a) adjusted means of standard length between morphs at hatching whilst accounting for differences in egg size, and b) slopes at first feeding (FF) for the anadromous population and six morphs of Arctic charr (FJ, Fljótaá; VS, Vatnshlíðarvatn silver; SV, Svínavatn; VB, Vatnshlíðarvatn brown; TP, Þingvallavatn planktivorous; TLB, Þingvallavatn large benthic; and GB, Galtaból benthic) with 95% confidence level intervals for predictions from the linear model. Data is based on mean egg and offspring size per female. Pairwise comparisons of slopes at FF were significantly different between the two Vatnshlíðarvatn morphs (VS and VB) and the benthic from lake Galtaból (both P <0.05, Table S2.3). The data points represent female means.*

2.5 Discussion

Developmental (phenotypic) plasticity has been proposed to affect evolution by facilitating adaptive change (West-Eberhard, 2003; Moczek et al., 2011), yet the role that egg size may play in the diversification of natural populations is only beginning to be understood (Pfennig & Martin, 2009; Martin & Pfennig, 2010; Leblanc et al., 2011, 2016; Smalås et al., 2017; Cogliati et al., 2018; Penney et al., 2018; Thorn & Morbey, 2018). Only a few studies have characterised intraspecific egg size variation in wild populations along a gradient of divergence (but see Räsänen & Hendry, 2008). Although the adoption of a new dietary resource can facilitate adaptive divergence (Smith & Skúlason, 1996; Roy et al., 2006; Pfennig & Martin, 2009), it is the degree of ancestral plasticity that guides such phenotypic differentiation (Ledon-Rettig et al., 2008; Wund et al., 2008; Schlichting & Wund, 2014). One potential source of such plasticity is egg size-mediated maternal effects. Here we studied an anadromous population and six morphs of Arctic charr along a gradient of divergence, from an anadromous (and putatively ancestral) population to different degrees of divergent landlocked populations. We found that landlocked populations invested in larger eggs, and that variation in egg size (after accounting for female length) decreased with increasing sympatric divergence, possibly reflecting canalisation of phenotypic variation. Additionally, we found that all landlocked populations hatched substantially later (ca. 420-460 DDs) than the anadromous Fljótaá population (ca. 390 DDs), with a planktivorous morph from lake Þingvallavatn also hatching at a larger size than the Fljótaá charr.

2.5.1 Variation in egg size

The causes and consequences of egg size differences are well documented in studies of life-history evolution of fishes (Hutchings, 1991, 2002; Kamler, 1992; Roff, 1992; Koops et al., 2003; Einum et al., 2004; Einum & Fleming, 2004; Rollinson & Hutchings, 2013). However, such maternal effects are rarely considered as an evolutionary force in the initial diversification of sympatric morphs (Pfennig & Martin, 2009), despite the growing wealth of literature documenting the pronounced effects of egg size on offspring phenotype (Leblanc et al., 2011, 2016; Cogliati et al., 2018; Penney et al., 2018; Self et al., 2018; Thorn & Morbey, 2018). We used an anadromous population from Fljótaá (FJ) as a model of the ancestral condition of Arctic charr. For absolute egg size, FJ and the two weakly diverged morphs from lake Vatnshlíðarvatn had the smallest egg sizes in comparison to the more strongly diverged morphs (Fig. 2.4a). After accounting for variation due to female, however, only the silver morph from lake Vatnshlíðarvatn had similar egg size to FJ, both of which had comparatively smaller eggs compared to all other morphs (Fig. 2.4b). Such a pattern might be reflective of a shared migratory reproductive strategy (although migratory distances most likely differ) between those two morphs. Indeed, Vatnshlíðarvatn silver (VS) retains it ancestral reproductive strategy of migrating to streams to spawn (Jónsson and Skúlason 2000). The production of smaller eggs in fluvial habitats where FJ and VS spawn suggests favourable environmental conditions based on the classic theory that there is a trade-off between offspring quantity and offspring quality (Smith & Fretwell, 1974; Fleming & Gross, 1989; Elgar, 1990; Einum & Fleming, 2000; Kinnisson et al. 2001). Previous studies found that migratory salmonids facing increased water velocities (Braun et al. 2013) and/or longer distances to spawning grounds (Fleming & Gross, 1989; Kinnisson et al. 2001) produced smaller eggs. Egg size also increased with higher temperature at the spawning ground (Braun et al., 2013). Thus suggesting that the smaller eggs in FJ and VS might be reflective of differences in life histories compared to the other morphs in this study, and possibly also differences in temperature. However, this latter hypothesis needs to be specifically tested.

We generally found that both morphs from lake Vatnshlíðarvatn (VS and the brown, VB) were relatively similar to the ancestral type in terms of mean egg size per female (Fig. 2.4, 7), as well as offspring size (Fig. 2.11). In absolute terms, within female variance in egg size ($CV_{eggSize}$) was much larger in VB and much smaller in GB than in the other morphs. However, after accounting for female size, variation in egg size declined in more diverged morphs (Fig. 2.8). Consistent with our prediction, a decrease in egg size variation was found with increasing magnitude of phenotypic divergence between sympatric morphs (Fig. 2.8b). This decrease in variation suggests that egg size might become canalised as morphs become more diverged and likely more specialised upon alternative resources, following canalisation of other phenotypic traits. Along this line, previous studies found that offspring from the more divergent Þingvallavatn population of Arctic charr had lower levels of morphological plasticity than offspring from the less diverged Vatnshlíðarvatn population, when reared under common garden conditions in the lab (Parsons et al., 2010, 2011).

Taken together, the wider range of egg sizes but overall smaller egg sizes in the ancestral population and the weakly diverged landlocked morphs, may indicate that phenotypic variation may be maintained in these morphs due to fluctuations in the late maturation/incubation environment. However, it is currently unknown as to how temperature during incubation of eggs varies among these morphs, and how the complexity of the habitats may also shape the observed egg size differences.

2.5.2 Variation in offspring phenotype

We found that developmental rates differed more between the anadromous Fljótaá population and the two Vatnshlíðarvatn morphs (brown and silver), than differences between the more strongly diverged morphs (Þingvallavatn large benthic and Þingvallavatn planktivorous). By retaining plasticity in developmental processes, organisms can alter various aspects of their development in accordance with changes in the environment. Disentangling whether such variation, or lack of variation, is due to canalisation or not can be difficult to infer from wild populations since stable laboratory-controlled conditions do not necessarily reflect those experienced in the wild. The size at which offspring start feeding was found to be attributed in part to egg size, with embryos from larger eggs from a highly diverged benthic morph from lake Galtaból (GB), starting to feed at a larger size compared to the weakly diverged sympatric morphs from lake Vatnshlíðarvatn (Fig. 2.11). Although we reared offspring in common garden conditions, differences in timing of first feeding between these two allopatric populations suggests that offspring have adapted to different environmental conditions. Furthermore, it would be interesting to examine GB's sympatric pelagic morph to determine whether the larger size at first feeding in GB had, at one point, facilitated the strong phenotypic and genetic divergence seen in this sympatric morph pair (Gíslason et al., 1999).

Egg size is often positively correlated with offspring size, and can be positively or negatively correlated with offspring developmental time (Thorpe et al., 1984; Johnson & Day, 2000; Valdimarsson et al., 2002; Bertram et al., 2009; Segers et al., 2012). Here we found no effects of egg size on hatching size (Fig. 2.11), but egg size did affect size at first feeding (Table 2.2), where egg size has been found to correlate with shape change at FF in the weakly diverged VS morph (*Paper IV*). Here we found that the significant morph x egg size interaction was attributable mostly to the highly diverged Galtaból benthic morph, whereby individuals from families with (on average) larger eggs started feeding at a larger size compared to the two weakly diverged morphs from lake Vatnshlíðarvatn (Fig. 2.11). Differences in size at first feeding can have considerable implications for ability to utilise available resources (Boubee & Ward, 1997; Dial et al., 2017). In cod (*Gadus morhua)* for instance, gape size of larvae at first feeding is strongly positively correlated with egg size (Knutsen & Tilseth, 1985). Egg size-mediated changes in feeding behaviour (Leblanc et al., 2011), combined with size-correlated constraints on diet choice (mediated by offspring size and associated mouth gape), can ultimately promote divergence in alternative resource use, especially in organisms with highly plastic trophic morphologies (Robinson & Wilson, 1996; Adams & Huntingford, 2004; Parsons et al., 2016).

2.5.3 Implications for the evolution of resource polymorphism

Resource polymorphism is the most common explanation for the co-existence of sympatric morphs in post-glacial lakes (Jonsson, 2001; Snorrason & Skúlason, 2004; Noakes, 2008). We studied sympatric morph pairs (Vatnshlíðarvatn VS and VB and Þingvallavatn TLP and TB) that vary in the degree of resource-related divergence. First, the two morphs from lake Vatnshlíðarvatn (VS and VB) overlap considerably in diet with both having a relatively benthic diet, with the only difference being that the silver morph becomes more of a generalist when food is abundant (Jónsson & Skúlason, 2000). These two morphs differ, however, in several life-history traits (growth, age and size at maturity; Jónsson & Skúlason, 2000), including spawning behaviour and location. The silver morph has retained the ancestral migratory life-history strategy of travelling in to streams to spawn, whereas the brown morph spawns in the lake at spawning grounds near the shore. Based on our findings, these two morphs also have pronounced differences in egg sizes and developmental timings, whereby the brown morph has relatively larger eggs (Fig. 2.4b) and higher variation in egg size (Fig. 2.8a), as well as slower development time to hatching than the silver morph (Fig. 2.9a). This suggests that the emergence and maintenance of the two Vatnshlíðarvatn morphs may primarily stem from differences in life-history traits, including maternal investment (Baker et al., 2005). In comparison, the sympatric morphs from lake Þingvallavatn show no differences in the aforementioned traits – although TP is larger at hatching – but these two morphs have very little overlap in diet (Malmquist et al., 1992) and are one of the classic examples of evolutionary diversification through resource polymorphism (Skúlason et al., 1989a; Malmquist et al., 1992; Skúlason & Smith, 1995).

Given the high degree of egg size variability in the VB morph and relatively reduced variance in the other landlocked morphs, different factors influencing per propagule maternal investment may be at play. First, it is possible that in the shallow lake Vatnshlíðarvatn – which is structurally simple and likely experiences numerous temperature fluctuations – females may favour a diversified bet-hedging strategy in maternal investment (Koops et al., 2003; Einum & Fleming, 2004). In contrast, in more stable environments a reduction in phenotypic variance may arise as a result of canalisation (Kuijper & Hoyle, 2015; Levis & Pfennig, 2018). However, the magnitude of divergence also depends upon how extensively sympatric morphs have adapted to alternative niches (Gíslason et al., 1999). One such reduction in phenotypic variance that we observe that can mediate differences in the plasticity of offspring traits is variation in egg size (Kaplan & Phillips, 2006; Pfennig & Martin, 2009; Cogliati et al., 2018; Penney et al., 2018). Canalisation of developmental processes contributing to the maintenance and divergence of phenotypic diversification may therefore manifest itself in egg/offspring size and associated developmental processess. Variation in egg size-mediated maternal effects may therefore prove to be a powerful driving force behind the evolution of resource polymorphisms (Pfennig & Martin, 2009; Martin & Pfennig, 2010).

2.5.4 Conclusion

This study is amongst the first to characterise variation in egg size, size and developmental timing of offspring along a gradient of divergence in a resource polymorphic fish. Although we found no evidence for the influence of egg size on developmental timing within populations/morphs, we did find a decrease in egg size variation in the landlocked morphs, providing some evidence for canalisation in more divergent Arctic charr morphs.

By taking advantage of the remarkable phenotypic plasticity in both sympatric and allopatric populations of Arctic charr in Iceland, our findings provide a solid foundation for future studies aiming to understand the underlying mechanisms and processes influencing the evolution of resource polymorphisms (Parsons et al., 2016; Ahi et al., 2018), and the role that maternal effects (egg size) might play in these processes. Maternal effects clearly provide a source of phenotypic variation in offspring traits, and understanding how egg size varies both within and across anadromous populations and landlocked morphs facilitates our understanding of mechanisms that might influence very early stages of origins of intraspecific diversity seen in post-glacial lakes today.

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2.7 Appendix

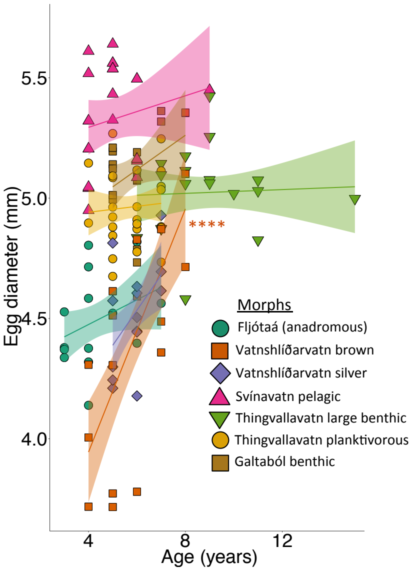

Figure S2.1 *Relationship between egg size and age of females. Only Vatnshlidaravtn brown had a slope that differed from zero (P <.0001). The anadromous population and all morphs except for Vatnshlíðarvatn silver differed from Vatnshlíðarvatn brown. See Table S2.1 for linear model results. Tukey adjusted P values are reported (P* < 0.05; P <.01**; P<.001***; P <.0001). Each point represents a single female.*

Table S2.1 *Sample sizes and summaries of offspring size data collected from females at post-fertlisation (PF) and eye stage (E) for measurements of egg size, as well as hatching and first feeding in an anadromous population and six morphs of Arctic charr in Iceland.*

					Egg size						**Hatching**					**First feeding**				
Morph	**♀ ID**	**♀ age**	**♀ FL**	**♂ ID**	**N**	**Cage**	**Mean egg size**	**CV (%)**	**PF DD**	**E DD**	**N**	**Cage**	**Mean SL**	**CV (%)**	**DD**	**N**	**Cage**	**Mean SL**	**CV (%)**	**DD**
FJ	292	–	33.3	297	15	1	4.70 ± 0.28	6.1	9	205	18	1	14.19 ± 0.36	2.54	388	10	1	21.35 ± 0.52	2.45	668
FJ	294	7	39.4	296	148	8	4.56 ± 0.15	3.3	9	205	80	4	13.81 ± 0.55	4.01	388	22	1	20.86 ± 0.72	3.46	707
FJ	295	6	35.8	297	17	1	4.40 ± 0.13	3.0	9	205	12	1	15.14 ± 0.43	2.84	417	11	1	20.29 ± 0.83	4.11	638
FJ	298	6	37.5	300	97	5	4.82 ± 0.21	4.3	9	201	65	3	13.83 ± 0.62	4.47	381	24	1	20.90 ± 0.91	4.35	707
FJ	299	4	31.5	300	65	2	4.14 ± 0.31	7.5	9	201	20	1	13.25 ± 0.58	4.38	405	10	1	19.10 ± 0.88	4.62	676
FJ	308	3	35.6	318	51	2	4.53 ± 0.40	8.8	10	199	20	2	15.71 ± 0.89	5.68	443	41	2	20.56 ± 0.78	3.77	649
FJ	309	4	35.8	316	53	3	4.58 ± 0.17	3.7	10	199	18	3	15.82 ± 0.64	4.04	441	20	1	20.12 ± 0.63	3.13	649
FJ	311	4	46	317	52	3	4.80 ± 0.12	2.4	10	199	18	3	16.54 ± 0.36	2.16	441	41	2	20.93 ± 0.53	2.51	649
FJ	312	4	40.2	318	46	4	4.32 ± 0.12	2.8	10	204	48	8	15.12 ± 0.43	2.85	444	41	2	19.18 ± 0.68	3.53	649
FJ	313	3	37.8	318	35	3	4.34 ± 0.11	2.5	10	204	24	4	15.45 ± 0.44	2.88	435	20	1	19.23 ± 0.90	4.69	649
FJ	314	4	41.5	317	100	6	4.71 ± 0.12	2.5	10	199	36	6	15.70 ± 0.53	3.37	446	20	1	20.79 ± 0.47	2.28	649
FJ	315	4	32.5	316	24	1	4.38 ± 0.17	3.9	10	204	20	2	15.55 ± 0.47	2.99	441	20	1	20.90 ± 0.73	3.48	649

FJ	338	3	37.3	344	19	2	4.38 ± 0.09	2.2	10	198	5	1	16.52 ± 0.50	3.01	443	14	1	20.63 ± 0.52	2.54	612
FJ	339	5	32.4	345	38	2	4.52 ± 0.21	4.6	10	198	20	2	15.85 ± 0.74	4.66	433	45	2	19.51 ± 0.69	3.52	617
FJ	343	3	35.5	345	50	4	4.37 ± 0.06	1.3	10	198	25	5	14.83 ± 0.70	4.70	422	20	1	19.41 ± 0.50	2.55	612
GB	38	5	19.9	51	18	2	4.99 ± 0.15	3.0	11	230						10	2	20.67 ± 0.51	2.44	618
GB	39	5	21.1	51	18	2	5.01 ± 0.18	3.7	11	230										
GB	40	5	22.3	52	18	2	5.14 ± 0.12	2.3	11	230						10	2	20.85 ± 0.49	2.33	618
GB	41	5	22.6	53	27	3	5.19 ± 0.10	1.9	11	230										
GB	42	7	22.3	50	27	3	5.36 ± 0.13	2.4	11	230						9	3	21.69 ± 1.18	5.46	618
GB	43	5	19.4	49	27	3	5.21 ± 0.10	1.8	11	230						9	3	20.45 ± 0.59	2.88	618
GB	44	5	21.5	52	27	3	5.12 ± 0.15	2.9	11	230						10	3	19.82 ± 0.77	3.88	618
GB	45	6	21.7	50	27	3	5.19 ± 0.15	2.8	11	230						9	3	20.78 ± 0.60	2.86	618
GB	46	7	23.6	51	27	3	5.32 ± 0.10	2.0	11	230						9	3	22.2 ± 0.48	2.18	618
GB	47	8	24.6	50	18	3	5.35 ± 0.18	3.4	11	230										
GB	48	6	20.5	49	27	3	5.07 ± 0.10	1.9	11	230						10	2	20.78 ± 0.43	2.05	618
GB	164	6	25.6	170	24	2	5.11 ± 0.14	2.7	10	226	26	2	15.38 ± 0.39	2.57	438	12	1	18.41 ± 0.72	3.93	619
GB	166	6	21.2	171	15	1	4.73 ± 0.10	2.0	10	226	17	1	14.84 ± 0.58	3.89	450	14	1	17.97 ± 1.63	9.05	619
GB	167	6	20.7	169	15	1	5.08 ± 0.18	3.5	10	226	18	1	14.31 ± 0.67	4.67	425	12	1	17.97 ± 0.60	3.36	619

GB	168	6	18.7	170	19	1	4.79 ± 0.16	3.3	10	226	13	1	14.05 ± 0.42	2.97	450	10	1	18.30 ± 0.30	1.66	619
SV	172	5	28.8	174	20	2	5.64 ± 0.29	5.1	10		19	1	16.31 ± 0.48	2.97	454					
SV	173	6	24.3	174	17	2	5.09 ± 0.08	1.6	10		11	1	16.69 ± 0.73	4.36	411					
SV	200	6	24.3	203	21	2	5.15 ± 0.16	3.2	9		16	1	14.69 ± 0.67	4.54	403					
SV	202	4	24.2	203	19	1	5.61 ± 0.34	6.1	9		17	1	14.29 ± 0.41	2.85	399					
SV	204	6	26.2	209	22	1	5.50 ± 0.23	4.1	9	203						11	1	22.54 ± 0.51	2.26	718
SV	205	5	27	208	22	1	5.56 ± 0.19	3.3	9	203					426	7	1	25.35 ± 0.70	2.74	697
SV	206	9	26.5	208	26	1	5.45 ± 0.17	3.2	9	203					407	10	1	22.28 ± 0.67	3.01	766
SV	207	6	25.5	209	26	1	5.16 ± 0.20	3.9	9	203					394	10	1	21.7 ± 0.52	2.40	718
SV	248	4	25.3	249	20	1	5.04 ± 0.12	2.4	10	202										
SV	250	4	24.9	259	22	1	4.95 ± 0.20	4.0	10											
SV	251	5	25.7	258	22	1	5.32 ± 0.12	2.3	10											
SV	252	5	25.9	259	22	1	5.45 ± 0.15	2.8	10											
SV	253	4	25.1	259	24	1	5.52 ± 0.23	4.1	10											
SV	254	5	26.4	260	20	1	5.54 ± 0.26	4.6	10	202										
SV	255	4	22.4	258	22	1	5.05 ± 0.16	3.2	10											
SV	256	4	28.5	260	22	1	5.32 ± 0.21	4.0	10	202										

SV	257	4	24.3	260	20	1	5.20 ± 0.25	4.9	10	206										
TLB	1	9	37.1	13	64	4	5.26 ± 0.24	4.5	14	207	7	3	17.15 ± 0.35	2.05	442	18	3	21.13 ± 0.67	3.17	680
TLB	2	8	30	12	59	3	5.11 ± 0.20	3.8	16	207	10	2	16.28 ± 0.43	2.65	443	16	2	20.40 ± 0.71	3.48	680
TLB	3	6	35.2	14	57	4	4.84 ± 0.20	4.1	14	207	15	5	15.88 ± 1.29	8.12	450	28	5	20.89 ± 1.04	4.95	677
TLB	5	9	37.8	15	61	4	5.08 ± 0.23	4.5	14	207	3	1	16.53 ± 0.34	2.07	439	30	5	20.71 ± 0.42	2.01	670
TLB	6	10	36.1	14	58	4	5.02 ± 0.19	3.8	15	207	15	5	16.19 ± 0.70	4.33	421	30	5	20.49 ± 0.58	2.83	672
TLB	7	9	32.3	15	56	3	5.06 ± 0.17	3.3	16	207	2	1	15.75 ± 0.16	1.04	441	28	3	21.20 ± 0.71	3.33	650
TLB	8	11	41.1	14	53	4	4.83 ± 0.21	4.4	14	207	6	2	16.47 ± 0.68	4.16	433	24	4	20.64 ± 0.84	4.06	677
TLB	9	7	29.7	12	57	3	5.14 ± 0.20	3.9	16	207	10	2	16.55 ± 0.24	1.44	441	17	3	20.41 ± 1.43	6.98	672
TLB	10	11	28.5	12	46	3	5.03 ± 0.14	2.8	16	207	8	1	16.31 ± 0.90	5.52	424	22	3	20.73 ± 0.62	3.00	665
TLB	11	11	37	15	64	4	5.07 ± 0.18	3.6	14	207	6	2	16.72 ± 0.34	2.01	436	12	2	21.42 ± 0.68	3.16	656
TLB	112	9	36.1	134	41	1	5.42 ± 0.15	2.8	12	220	10	2	12.92 ± 0.73	5.65	446					
TLB	115	15	49.9	134	18	1	5.00 ± 0.28	5.7	12	220	30	5	13.87 ± 1.04	7.53	434					
TLB	116	8	40.3	132	59	2	5.17 ± 0.25	4.8	12	220	15	3	14.56 ± 1.52	10.43	445	15	1	19.51 ± 0.76	3.92	643
TLB	117	8	42.8	131	19	1	5.06 ± 0.24	4.7	12	220										
TLB	119	7	39.1	134	17	1	4.87 ± 0.14	2.8	12	220	16	2	15.91 ± 0.97	6.08	425	14	1	18.69 ± 0.77	4.14	643
TLB	124	7	35.9	130	41	1	5.10 ± 0.18	3.5	12	220	10	2	15.43 ± 0.58	3.77	433	10	1	20.16 ± 0.73	3.64	643

TLB	128	8	40	129	18	1	4.58 ± 0.31	6.7	12	220	11	2	15.22 ± 0.62	4.10	432	16	1	19.42 ± 2.17	11.16	660
TP	82	7	21	104	18	3	4.73 ± 0.21	4.5	13	226	9	3	16.05 ± 0.85	5.33	442	9	3	21.84 ± 1.04	4.75	654
TP	83	6	21	111	41	4	5.12 ± 0.25	4.9	14	226	9	3	15.94 ± 2.09	13.09	461	9	3	21.30 ± 0.76	3.59	634
TP	84	7	19.5	103	29	4	5.11 ± 0.45	8.9	12	226	9	3	16.64 ± 0.80	4.78	453	9	3	22.14 ± 0.82	3.69	642
TP	85	5	19.2	105	23	4	5.08 ± 0.23	4.5	16	226	9	3	17.30 ± 0.62	3.56	457	9	3	22.26 ± 0.61	2.73	638
TP	86	7	20.4	104	29	4	4.88 ± 0.20	4.0	14	226	9	3	17.32 ± 0.64	3.67	457	9	3	22.16 ± 0.60	2.70	642
TP	88	6	19.6	102	15	3	4.91 ± 0.21	4.3	16	226	9	3	16.44 ± 0.49	2.96	453	9	3	21.82 ± 0.59	2.71	654
TP	89	7	20.1	102	15	3	5.05 ± 0.32	6.4	14	226	6	2	17.12 ± 0.48	2.81	453	6	2	22.04 ± 0.87	3.96	654
TP	91	5	20	105	18	3	4.96 ± 0.10	1.9	14	226	11	3	17.19 ± 1.26	7.33	461	9	3	22.07 ± 1.27	5.76	654
TP	92	5	20.1	105	28	4	4.92 ± 0.24	4.9	14	226	9	3	16.92 ± 0.83	4.90	453	9	3	22.10 ± 1.05	4.74	636
TP	99	4	19.6	103	23	4	4.90 ± 0.24	5.0	16	226	9	3	16.08 ± 0.46	2.86	453	10	2	21.15 ± 0.90	4.24	654
TP	240	5	21.2	239	16	1	5.09 ± 0.11	2.2	9	263	13	1	16.23 ± 0.44	2.69	408	14	1	20.97 ± 1.00	4.78	681
TP	241	6	20.6	239	16	1	4.96 ± 0.18	3.7	9	263	15	1	16.26 ± 0.69	4.24	431					
TP	250	5	22.5	245	22	2	4.88 ± 0.31	6.3	9	263	6	1	15.61 ± 0.87	5.56	448	11	1	20.87 ± 0.71	3.39	672
TP	251	5	21.2	249	16	1	5.27 ± 0.22	4.1	9	263	8	1	17.18 ± 0.33	1.91	431	11	1	21.02 ± 0.96	4.58	672
TP	252	4	22.6	245	17	1	5.15 ± 0.18	3.4	9	263	14	1	15.44 ± 0.61	3.97	408	12	1	21.31 ± 0.88	4.13	719
TP	253	7	22	249	17	1	5.03 ± 0.11	2.2	9	263	12	1	16.39 ± 0.46	2.78	431	11	1	20.28 ± 0.68	3.37	672

TP	319	6	21	337	19	1	4.78 ± 0.19	3.9	5	200	20	1	17.13 ± 0.71	4.14	450	21	1	21.32 ± 0.86	4.04	630
TP	320	5	19.9	335	20	1	4.67 ± 0.21	4.5	5	200	16	1	16.39 ± 0.74	4.52	457	22	1	19.64 ± 1.03	5.26	630
TP	323	5	18.5	336	16	1	4.75 ± 0.35	7.4	5	200	9	1	16.44 ± 0.53	3.22	457	21	1	20.38 ± 1.13	5.53	630
TP	324	7	23.7	334	15	1	5.25 ± 0.15	2.9	5	200	16	1	17.96 ± 0.64	3.56	453	20	1	22.22 ± 1.21	5.44	630
TP	325	6	19.7	337	15	1	4.87 ± 0.23	4.7	5	200	18	1	16.78 ± 0.58	3.44	453	22	1	20.89 ± 0.65	3.11	630
TP	329	6	20.2	335	17	1	4.89 ± 0.19	3.8	5	200	16	1	17.22 ± 0.67	3.92	453	20	1	21.08 ± 0.66	3.14	630
TP	330	5	22.9	333	18	1	4.84 ± 0.08	1.7	5	200	9	1	17.24 ± 0.91	5.26	457	20	1	20.84 ± 0.83	3.97	630
TP	332	7	21.3	333	20	1	4.88 ± 0.23	4.8	5	200	16	1	17.31 ± 0.43	2.49	446	20	1	20.36 ± 1.25	6.16	630
VB	16	5	17.6	34	48	4	4.61 ± 0.28	6.1	27	234	18	3	14.65 ± 1.21	8.24	464	18	3	19.04 ± 1.29	6.80	657
VB	17	7	17	34	32	3	4.87 ± 0.50	10.3	27	234	9	2	14.64 ± 1.13	7.75	466	20	2	18.81 ± 2.23	11.84	683
VB	18	5	14.5	36	31	3	3.77 ± 0.29	7.7	27	234	12	2	14.25 ± 1.40	9.84	456	15	2	18.05 ± 1.61	8.92	654
VB	19	8	18.9	36	17	4	4.71 ± 0.17	3.5	27	234										
VB	23	5	16.3	32	19	3	3.72 ± 0.16	4.2	27	234	12	2	13.49 ± 0.61	4.53	462	20	2	16.04 ± 1.90	11.86	658
VB	24	6	15.2	33	32	3	3.78 ± 0.24	6.4	27	234	12	2	13.22 ± 0.37	2.78	462	20	2	17.56 ± 1.80	10.26	632
VB	25	6	18.6	35	38	4	4.83 ± 0.37	7.6	27	234	18	3	15.09 ± 1.08	7.17	460	18	3	17.65 ± 1.45	8.23	659
VB	26	4	13.7	32	32	3	3.72 ± 0.14	3.7	27	234	12	2	15.06 ± 1.93	12.78	458	17	2	15.86 ± 1.24	7.85	624
VB	28	8	20.6	35	47	4	5.36 ± 0.46	8.6	27	234	18	3	15.79 ± 1.09	6.90	463	18	3	18.44 ± 1.84	9.97	665

VB	30	4	14.7	32	22	3	4.31 ± 0.54	12.6	27	234	12	2	14.46 ± 1.42	9.79	464	19	2	17.14 ± 0.99	5.77	666
VB	31	4	14.2	35	41	4	4.01 ± 0.29	7.1	27	234	18	3	15.14 ± 1.14	7.50	458	18	3	16.48 ± 1.56	9.44	670
VB	135	5	17.6	160	19	2	4.51 ± 0.18	4.0	12	217	17	1	15.00 ± 0.76	5.05	470	25	1	18.14 ± 0.70	3.87	639
VB	143	6	24.8	156	72	3	4.59 ± 0.21	4.5	12	217	28	2	13.29 ± 0.68	5.12	386	24	1	18.11 ± 1.95	10.77	639
VB	148	7	26.4	161	17	2	4.49 ± 0.21	4.7	12	217										
VB	149	7	17.2	163	53	2	4.36 ± 0.10	2.2	12	230	19	1	14.51 ± 0.87	6.02	441	9	1	17.53 ± 0.63	3.60	639
VB	150	8	24.1	158	24	2	5.10 ± 0.43	8.5	12	217	26	2	15.19 ± 1.07	7.02	429	25	1	19.66 ± 1.42	7.24	639
VB	152	5	15.7	161	96	2	4.31 ± 0.13	3.0	12	217	14	1	14.31 ± 0.51	3.55	442	17	1	17.38 ± 1.22	7.04	639
VS	54	5	23.3	73	22	3	4.57 ± 0.19	4.2	12	222	10	2	14.83 ± 1.43	9.63	433	13	2	19.17 ± 0.96	5.03	617
VS	56	6	24.5	73	27	3	4.18 ± 0.13	3.2	12	222	10	2	15.33 ± 0.70	4.57	435	11	1	18.5 ± 0.86	4.62	644
VS	65	7	27.8	74	49	4	4.69 ± 0.13	2.7	12	222	18	3	15.10 ± 0.86	5.67	435	18	3	18.92 ± 0.77	4.06	617
VS	67	5	21.7	74	36	3	4.30 ± 0.11	2.6	12	222	16	2	14.94 ± 0.74	4.98	431	20	2	18.21 ± 0.46	2.51	642
VS	68	7	22.5	72	33	4	4.61 ± 0.21	4.5	12	222	15	3	15.09 ± 0.60	3.98	427	15	2	19.19 ± 0.77	4.00	650
VS	69	5	22	73	32	3	4.21 ± 0.11	2.6	12	222	16	2	14.90 ± 0.55	3.68	429	20	2	18.78 ± 0.98	5.21	641
VS	71	6	22.3	74	26	3	4.45 ± 0.18	4.0	12	222	16	2	15.18 ± 0.64	4.21	440	20	2	19.48 ± 0.45	2.31	634
VS	175	6	25	186	21	2	4.50 ± 0.17	3.8	10	217	5	1	14.21 ± 0.27	1.90	440	21	1	18.37 ± 0.53	2.87	626
VS	176	7	25	188	16	1	4.93 ± 0.33	6.7	10	217	15	1	13.86 ± 0.50	3.64	383	22	1	18.73 ± 0.90	4.78	639

VS	180	6	25	185	63	3	4.44 ± 0.18	4.0	10	217	31	2	13.20 ± 0.50	3.79	391	20	1	18.55 ± 0.82	4.41	655
VS	182	5	23	187	57	2	4.24 ± 0.18	4.2	10	217	16	1	12.94 ± 0.71	5.47	391	26	1	17.33 ± 0.99	5.73	655
VS	184	6	24.2	187	16	1	4.61 ± 0.17	3.6	10	217	7	1	14.29 ± 0.38	2.63	403	22	1	19.07 ± 0.76	4.00	639
VS	190	6	27.5	195	62	2	4.63 ± 0.18	4.0	10	220	32	1	13.82 ± 0.92	6.68	398	20	1	18.46 ± 0.74	4.01	639
VS	194	5	24	195	16	1	4.81 ± 0.28	5.8	10	217	14	1	15.20 ± 0.66	4.35	416	25	1	19.57 ± 0.89	4.56	626

♀, female; FL, fork length; ID, identification; N, number of offspring; CV, coefficient of variation; DD, degree days; SL, standard length.

***Table S2.2** Timing of development (using degree days, DD) for hatching and first feeding for when: a) the average of individuals from each female (♀) reach a developmental stage; and b) when 50% of the cage, and then average of each female, reaches a particular developmental stage in the anadromous population and four morphs of Arctic charr.*

a)

Morph	♀	Hatching (N)	First feeding (N)	Hatching (DD)	First feeding (DD)
TLB	1	30	27	442	680
	2	28	19	443	680
	3	29	24	450	677
	5	31	30	439	670
	6	28	25	421	672
	7	29	20	441	650
	8	20	19	433	677
	9	25	17	441	672
	10	27	24	424	665
	11	30	23	436	656
VB	16	27	27	464	657
	17	17	12	466	683
	18	19	16	456	654
	23	18	16	462	658
	24	18	21	462	632
	25	24	19	460	659
	26	20	12	458	624
	28	27	24	463	665
	30	10	8	464	666
	31	25	21	458	670
VS	54	7	8	433	617
	56	20	10	435	644

	65	30	22	435	617
	67	20	10	431	642
	68	13	9	427	650
	69	20	14	429	641
TM	83	20	7	461	634
	84	20	4	453	642
	85	20	12	457	638
	86	20	10	457	642
	92	20	11	453	636

b)

Morph	♀	Hatching (DD)	First feeding (DD)
TLB	116	445	643
	119	425	643
	124	433	643
	128	432	660
FJ	292	388	668
	294	413	702
	295	417	638
	298	413	709
	299	405	677

Table S2.3 *Pairwise comparisons of significant contrasts only for a) pairwise comparisons of adjusted means, and b) comparisons of slopes examining how egg size, and developmental time differences between an anadromous population (encompassed within 'morph') and six morphs of wild Arctic charr (Salvelinus alpinus).*

a) Pairwise comparisons of adjusted means

Egg size ~ morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	SV	-0.83	0.07	216	-11.95	<.0001
FJ	TLB	-0.52	0.07	216	-8	<.0001
FJ	TP	-0.46	0.06	216	-7.64	<.0001
FJ	GB	-0.6	0.07	216	-9	<.0001
VB	SV	-0.92	0.07	216	-13.55	<.0001
VB	TLB	-0.61	0.06	216	-9.63	<.0001
VB	TP	-0.55	0.06	216	-9.43	<.0001
VB	GB	-0.69	0.07	216	-10.61	<.0001
VS	SV	-0.82	0.07	216	-11.62	<.0001
VS	TLB	-0.51	0.07	216	-7.7	<.0001
VS	TP	-0.45	0.06	216	-7.32	<.0001
VS	GB	-0.59	0.07	216	-8.69	<.0001
SV	TLB	0.31	0.07	216	4.51	<.001
SV	TP	0.37	0.06	216	5.81	<.0001
SV	GB	0.23	0.07	216	3.27	0.021

Timing of hatching ~ morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	VS	-35.73	7.23	40	-4.94	≤0.0001
FJ	VB	-65.29	6.54	40	-9.98	<.0001
FJ	TP	-44.89	6.54	40	-6.86	<.0001
FJ	TLB	-40.08	6.22	40	-6.44	<.0001
VS	VB	-29.55	6.17	40	-4.79	<0.001

VB	TP	20.40	5.34	40	3.82	0.004
VB	TLB	25.21	4.95	40	5.10	≤0.0001

Timing of first feeding ~ morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	VS	43.68	12.30	40	3.55	0.008

Timing of hatching ~ egg size + morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	VB	-64.97	6.75	39	-9.62	<.0001
FJ	VS	-35.6	7.34	39	-4.85	<.001
FJ	TLB	-40.75	6.92	39	-5.89	<.0001
FJ	TP	-45.62	7.31	39	-6.24	<.0001
VB	VS	29.37	6.29	39	4.67	<.001
VB	TLB	24.22	6.54	39	3.71	0.006

Timing of first feeding ~ egg size + morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	VS	42.16	12.07	39	3.49	≤0.01

Size at hatching ~ morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	TP	-1.54	0.28	85	-5.44	<.0001
VS	TP	-2.20	0.29	85	-7.63	<.0001
VS	TLB	-1.24	0.31	85	-3.96	0.003
VB	TP	-2.15	0.28	85	-7.62	<.0001
VB	TLB	-1.19	0.31	85	-3.87	0.004
TP	TLB	0.96	0.28	85	3.46	0.014
TP	GB	2.05	0.46	85	4.42	<0.001

Size at hatching ~ egg size + morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	TP	-1.28	0.32	84	-4.01	0.002
VB	TP	-1.83	0.34	84	-5.39	<.0001
VS	TP	-1.95	0.32	84	-6.04	<.0001
TLB	TP	-1	0.28	84	-3.65	0.008
TP	GB	2.03	0.46	84	4.43	0.001

Size at first feeding ~ morph

Contrast		Estimate	SE	Df	*t*	*P*
FJ	VS	1.51	0.35	90	4.32	0.001
FJ	VB	2.52	0.34	90	7.34	<.0001
FJ	SV	-2.72	0.53	90	-5.13	<.0001
FJ	TP	-1.06	0.31	90	-3.38	0.018
VS	SV	-4.23	0.53	90	-7.92	<.0001
VS	TP	-2.57	0.32	90	-8.04	<.0001
VS	TLB	-1.68	0.36	90	-4.71	0.000
VS	GB	-1.25	0.37	90	-3.38	0.018
VB	SV	-5.24	0.53	90	-9.89	<.0001
VB	TP	-3.58	0.31	90	-11.45	<.0001
VB	TLB	-2.69	0.35	90	-7.68	<.0001
VB	GB	-2.26	0.36	90	-6.21	<.0001
SV	TP	1.66	0.51	90	3.26	0.026
SV	TLB	2.55	0.53	90	4.78	0.000
SV	GB	2.98	0.54	90	5.47	<.0001
TP	GB	1.32	0.34	90	3.92	0.003

b) Comparisons of slopes

*Egg size ~ female FL * morph*

Contrast		Estimate	SE	Df	*t*	*P*
FJ	VB	-0.07	0.01	209	-4.56	0
VB	TLB	0.1	0.01	209	7.84	<.0001

*Egg size variation ~ female FL * morph*

Contrast		Estimate	SE	Df	*t*	*P*
FJ	SV	0.03	0.01	209	3.4	0.014
FJ	TLB	0.04	0.01	209	3.71	0.005
FJ	TP	0.06	0.01	209	4.63	0
FJ	GB	0.05	0.01	209	3.84	0.003
VB	TLB	0.03	0.01	209	3.17	0.028
VB	TP	0.05	0.01	209	4.21	0.001
VB	GB	0.03	0.01	209	3.32	0.018
VS	TP	0.03	0.01	209	3.04	0.042

*Size at first feeding ~ egg size * morph*

Contrast		Estimate	SE	Df	*t*	*P*
VB	GB	-4.42	1.24	83	-3.55	0.011
VS	GB	-4.64	1.49	83	-3.11	0.039

SE, standard error; Df, degrees of freedom; *t, t*-value

3 Paper II

Gene expression in the phenotypically plastic Arctic charr (*Salvelinus alpinus*): A focus on growth and ossification at early stages of development

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RESEARCH

WILEY EVOLUTION & DEVELOPMENT

Gene expression in the phenotypically plastic Arctic charr (Salvelinus alpinus): A focus on growth and ossification at early stages of development

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Gene expression during development shapes the phenotypes of individuals. Although embryonic gene expression can have lasting effects on developmental trajectories, few studies consider the role of maternal effects, such as egg size, on gene expression. Using qPCR, we characterize relative expression of 14 growth and/or skeletal promoting genes across embryonic development in Arctic charr (Salvelinus alpinus). We test to what extent their relative expression is correlated with egg size and size at early life-stages within the study population. We predict smaller individuals to have higher expression of growth and skeletal promoting genes, due to less maternal resources (i.e., yolk) and prioritization of energy toward ossification. We found expression levels to vary across developmental stages and only three genes (Mmp9, Star, and Sgk1) correlated with individual size at a given developmental stage. Contrary to our hypothesis, expression of Mmp9 and Star showed a non-linear relationship with size (at post fertilization and hatching, respectively), whilst Sgk1 was higher in larger embryos at hatching. Interestingly, these genes are also associated with craniofacial divergence of Arctic charr morphs. Our results indicate that early life-stage variation in gene expression, concomitant to maternal effects, can influence developmental plasticity and potentially the evolution of resource polymorphism in fishes.

1 | INTRODUCTION

The ability of individuals to respond to environmental heterogeneity largely depends upon changes in gene expression, framed by physiological, behavioral, and anatomic constraints (Taugbøl, Arntsen, Østbye, & Vøllestad, 2014). Such interactions between genes and environment are most often realized through development, thus providing phenotypic variation upon which natural selection can act (e.g. West-Eberhard, 2005).

During early stages of development, an organism's phenotype tends to be malleable (Hoverman & Relyea, 2007). The effects of such developmental plasticity often persist through to the adult phenotype (Gabriel, 2006; Godfrey, Lillycrop, Burdge, Gluckman, & Hanson, 2007; Holoch & Moazed, 2015) and may facilitate rapid evolutionary changes at the population level (Fusco & Minelli, 2010; Price, Qvarnstrom, & Irwin, 2003). One way to further our understanding of divergence of developmental trajectories is to examine the underlying changes in gene expression and deducing the related molecular processes (Politis et al., 2017; Schneider & Meyer, 2017). Examining patterns of gene expression across developmental stages can therefore provide a powerful approach to unveiling mechanisms involved in phenotypic diversification, and ultimately its role in evolution (Aubin-Horth & Renn, 2009; Schneider, Li, Meyer, & Gunter, 2014).

Maternal effects, here defined as the causal influence of the maternal genotype or phenotype on offspring phenotype and fitness (Wolf & Wade, 2009), reflect a form of transgenerational effects (Kuijper & Hoyle, 2015; Uller, 2008). Maternal effects can have a strong impact on phenotypic development (Badyaev, 2008; Mousseau & Fox, 1998) and influence evolution at ecological time scales (see Räsänen & Kruuk, 2007; Uller, 2008). In many organisms, maternal allocation of resources (i.e., size and composition of propagules) is an important determinant of development at early life-stages (Bernardo, 1996; Mousseau & Fox, 1998).

In fish, egg size is not only heritable (Kinnison, Unwin, Hendry, & Quinn, 2001; Su, Liljedahl, & Gall, 1997), but can vary both within and among females and is also influenced by the environment the mother experiences, as well as maternal age and size (Chambers & Leggett, 1996; Cogliati, Unrein, Stewart, Schreck, & Noakes, 2018; Leblanc, Benhaïm, Hansen, Kristjánsson, & Skúlason, 2011; Segers & Taborsky, 2012). In turn, egg size can strongly affect behavior and fitness of offspring (Benhaïm, Skúlason, & Hansen, 2003; Leblanc et al., 2011; Leblanc, Kristjánsson, & Skúlason, 2016), with smaller embryos often developing faster than larger embryos (Cogliati et al., 2018; Leblanc et al., 2011; Valdimarsson, Skúlason, & Snorrason, 2002). Despite the widely accepted view that egg size can affect offspring fitness (Bernardo, 1996; Mousseau & Fox, 1998), only one study has reported a link between egg size and offspring gene expression in fishes. Segers et al. (2012) found the expression levels of the growth hormone receptor (GHR) to be lower in smaller than larger cichlid embryos (originating from small and larger eggs, respectively), a pattern that was reversed after hatching. Such egg size-dependent gene expression demonstrates the potential of maternal effects to influence developmental plasticity.

Finally, the model of evolution of resource polymorphism (alternative phenotypes utilising different resources; Smith & Skúlason, 1996) proposes that phenotypic plasticity is important during early stages of diversification (e.g., Schneider & Meyer, 2017; Skúlason, Snorrason, & Jonsson, 1999), and this may involve egg size-mediated maternal effects (Benhaïm et al., 2003; Cogliati et al., 2018; Leblanc et al., 2011). Resource polymorphism is common in fishes, such as Arctic charr (Salvelinus alpinus), that inhabit young freshwater systems of the Northern hemisphere – likely due to lack of predation and increased intraspecific competition for habitats and food resources promoting character release (Skúlason & Smith, 1995). Although the role of developmental plasticity in diversification has been less studied than the role of genetic and ecological factors, it is becoming increasingly recognised as a key factor in the generation of polymorphism (Pfennig et al., 2010; West-Eberhard, 2003). In this vain, Ahi et al. (2014) found gene expression networks that are associated with craniofacial divergence in developing heads of benthic and pelagic Arctic charr morphs. This gene expression variation is also reflected – to some extent – in morphology (Kapralova et al., 2015), and could explain variable timing of ossification during embryonic development in the offspring of benthic and pelagic morphs of Arctic charr (Eiríksson, Skúlason, & Snorrason, 1999).

Building upon previous findings, we aim to further understanding of patterns of gene expression during early development and how this variation relates to individual size – an important fitness trait – using a so called "brown" Arctic charr morph (BM) from lake Vatnshlíðarvatn, NW Iceland (Jónsson & Skúlason, 2000). This morph is a good candidate for studies of maternal effects and ontogenetic variation in gene expression because: 1) egg size is highly variable within and between females of similar sizes (Beck et al., in prep) and 2) it is believed to be at the early stages of divergence from another (so called "silver") morph in this lake (Jónsson & Skúlason, 2000; Parsons, Skúlason, & Ferguson, 2010).

We tested the mRNA levels of 14 growth and/or skeletal promoting genes from post fertilization to first feeding, relative to two reference genes. We will refer to these relative differences in gene expression simply as gene expression. The growth-related candidate genes were selected based on a literature search, whilst skeletal-related genes were chosen to expand upon previous findings (Ahi et al., 2014; see below for further details). We studied patterns of gene expression across four developmental stages to test whether expression of growth-related genes is higher in larger offspring (Segers et al., 2012) and whether, in contrast, expression of genes related to skeletogenesis is higher in smaller offspring (Ahi et al., 2014). These predictions are based on the hypotheses that larger individuals (i.e., originating from larger eggs) invest more energy toward growth, whilst smaller offspring (i.e., originating from smaller eggs) invest more in ossification (Eiríksson et al., 1999; Segers et al., 2012). The latter is expected because lower maternal investment in yolk should result in embryos prioritizing development of essential skeletal structures such as those related to external feeding. We assume that differences in mRNA levels at post fertilization stage – before the embryos genome has been activated (i.e., before the mid-blastula transition, MBT; Kane

& Kimmel, 1993; Nagasawa, Fernandes, Yoshizaki, Miwa, & Babiak, 2013) – will reflect maternally deposited mRNA in the egg. Due to the strong correlation between egg size and offspring size (Leblanc et al. unpublished data, see below), we will hereafter refer to “size” as encompassing both pre-hatched and hatched offspring. We document size-correlated changes in gene expression at the individual level and across multiple developmental stages, providing a powerful approach to determine the importance of offspring size for developmental variation in gene expression.

2 | MATERIALS AND METHODS

2.1 | Study system

Vatnshlíðarvatn is a physically simple, small (~70 ha) and shallow (mean depth 2-3 m) lake (Jónsson & Skúlason, 2000). Arctic charr is the only fish species in the lake and has diverged into two morphs (silver and brown). The brown morph has a relatively benthic shape, with a deeper body, and a more pronounced subterminal mouth (Parsons, Sheets, Skúlason, & Ferguson, 2011). In this morph, egg diameter and standard length of the embryos at hatching and first feeding are strongly correlated (at hatching: $R^2 = 0.96$,; at first feeding: $R^2 = 0.97$; $N_{(females)} = 12$ and $N_{(offspring)} >= 168$; both $P < 0.001$; Leblanc et al., unpublished data).

Mature females (N = 7) and males (N = 5) were caught using gill nets (15–19 mm) in early September 2014 and 2015. All females were mated to a single male, but a subset of (N = 3) females shared the same male. Although this design causes variation in the relatedness of offspring, it was used to minimize potential direct genetic effects. Eggs and milt were collected and mixed in the field, where fertilized eggs were allowed to water-harden before transport to Hólar University College's aquaculture facilities in Verið, Sauðárkrókur. Fishing in Vatnshlíðarvatn was done with permission obtained from the owner of the land (N.B. ethics’ committee approval is not needed for regular or scientific fishing in Iceland; the Icelandic law on Animal protection, Law 15/1994, last updated with Law 157/2012). Sampling was performed by Hólar University College Aquaculture Research Station (HUC-ARC) personnel. HUC-ARC has an operational license according to Icelandic law on aquaculture (Law 71/2008), which includes clauses of best practices for animal care and experiments.

Embryos were reared in plastic cages (6.5 cm × 6.5 cm × 5 cm) with 2 cm × 2 cm holes at the front and rear. The holes and the bottom of the cage were covered with mesh to enable good oxygenation. Cages were placed in a shelf incubator system (MariSource 8-tray Vertical Incubator) with a constant flow of 95% recycled water. For most families, a single cage was used, but when eggs were numerous they were split into different cages to assure only one layer of eggs per cage for sufficient oxygenation. To mimic natural conditions, embryos were reared in darkness at 3.5–4.5 ºC. Temperature was manually checked on a daily basis and recorded using HOBO loggers (to the nearest 0.01 °C) four times daily. Dead individuals (opaque eggs) were manually removed every 2 days to prevent fungal growth. An accumulative temperature estimate (degree days, DD; Pruess, 1983) was used to determine the approximate time by which developmental stages would occur (see below).

2.2 | Study design

Gene expression of Arctic charr was estimated at four developmental stages: 1) post fertilization (PF; 27DD), before the MBT (Kane & Kimmel, 1993); 2) eye stage (E; ~200 DD), when eye lenses are formed and retinas pigmented; 3) hatching (H, ~400 DD), when individuals have hatched but still rely on nutrition from the yolk sac; and 4) first external feeding (FF, ~600 DD; Ballard, 1973). Sampling at each of the four stages occurred when approximately 50% of individuals within each family reached that particular stage.

At PF and E stages, individuals were visually sampled (Leblanc et al., 2011) based on their relative size (small, medium, and large) within a given clutch (Table 1). Equal numbers of individuals were taken for each relative size class and female to ensure a good representation of all egg sizes. At H and FF, individuals were chosen randomly since size selection was not feasible. A total of 5–8 individuals/family/developmental stage were collected for gene expression analyses (Table 1) and euthanised using 2-phenoxyethanol (Pounder, Mitchell, Thomson, Pottinger, & Sneddon, 2017). All individuals (eggs, or left side of H and FF embryos) were digitally photographed (Canon EOS 650D) with a length scale, and measured for egg diameter or standard length (SL; Leblanc et al., 2016) using the program Fiji (Schindelin et al., 2012). Individual egg size was determined at PF and E stages by taking the average of four diameter measurements for each egg. All size measurements were taken to the nearest 0.001 mm. For egg size variation for additional females (not used in this study), see Table S1. Individuals from all developmental stages were then stored in an RNA stabilizing buffer (De Wit et al., 2012) and pre-incubated at room temperature before storage (−20 ºC).

2.3 | RNA extraction and cDNA synthesis

Gene expression was measured using qPCR for the whole embryo at PF, E, and H stages (see Table 1 for sample sizes). Due to their large size, FF individuals were decapitated behind the pectoral fin and RNA extracted from the head and body separately. However, only the head was included in this study as much of the phenotypic variation in Arctic charr is

TABLE 1 Female and offspring measurements ± standard deviation (SD) of Arctic charr (Salvelinus alpinus) from Lake Vatnshlíðarvatn brown morph, sampled across four developmental stages: post fertilization (PF); eye stage (E); hatching (H); first feeding (FF)

Female				Offspring							
				PF		E		H		FF	
Female identity	Fork length (cm)	Weight after stripping (g)	Weight before stripping (g)	Mean egg diameter (mm)	N	Mean egg diameter (mm)	N	Mean SL (mm)	N	Mean SL (mm)	N
16	17.6	58.5	na	4.6 ± 0.07	5	4.5 ± 0.11	8	15.1 ± 0.59	7	17.9 ± 0.51	5
18	14.5	35	na	3.8 ± 0.1	6	3.7 ± 0.16	4	13.4 ± 0.36	6	16.6 ± 0.44	7
24	15.2	36.5	40	3.8 ± 0.08	6	3.6 ± 0.1	3	13.4 ± 0.32	5	16.4 ± 0.4	4
25	18.6	66	85	4.9 ± 0.07	5	4.9 ± 0.04	3	15.9 ± 0.27	6	19.1 ± 0.31	5
28	20.6	89	110	5.4 ± 0.11	5	5.5 ± 0.1	6	17 ± 0.28	6	20.5 ± 0.3	8
30	14.7	32	na	3.9 ± 0.1	6	4 ± 0.11	5	14.4 ± 0.34	6	17.7 ± 0.7	7
31	14.2	30	40	4 ± 0.08	6	4 ± 0.08	6	14 ± 0.33	4	16.2 ± 0.69	6

Sample sizes differ due to loss of samples during RNA extractions. Degree days were the same within stages (PF = 27; E = 242; H = 473; and FF = 701). na = data not available. Mean individual size (SL, standard length) and standard deviation (SD) of offspring used in this study were measured for all females. Samples sizes (N) represent all individuals for which RNA was successfully extracted.

associated with trophic morphology, as well as large body size being reflected by large head size (Kapralova et al., 2015).

Tissues were homogenized using Bead Beater (Biospec) and total RNA extracted using TRI reagent (Sigma–Aldrich, St Louis, MO). RNA extraction and cDNA synthesis were conducted according to Ahi et al. (2013). RNA was precipitated using isopropanol, washed with ethanol and air-dried. The RNA pellet was resuspended in RNase-free water and the RNA yields quantified using NanoDrop ND-1000 UV/VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE). The samples were treated with DNase I (New England, Biolabs, Ipswich, MA) to remove any contaminating DNA. A subset of extracted RNAs were electrophoresed on agarose gels to test RNA quality. Single stranded cDNA was synthesized from 1 µg of total RNA, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. cDNA was consequently diluted in nuclease-free water in preparation for qPCR.

Fourteen genes were chosen for analyses of early life-stage gene expression based on their previously demonstrated involvement in growth or skeletal development (see below). Two validated early developmental Arctic charr reference genes (Actb and Ef1a) were chosen for qPCR data normalisation based on Ahi et al., (2013). The following target growth-promoting genes were selected, together with components of the glucocorticoid signaling pathway (mediating energy mobilization and stress responses): Star is associated with steroid and cortisol synthesis and involved in the stress response of fishes (Alsop & Vijayan, 2008); Igf1 and Igf2 are insulin-like growth factors (IGFs) that play important roles in regulating growth and development in vertebrates (Greene & Chen, 1999); Nr3c1–here called Gr –, is a glucocorticoid receptor and mediator of corticosteroid signalling, highly abundant in ovulated oocytes and crucial for later skeletogenesis, particularly in the craniofacial skeleton (Pikulkaew et al., 2011); Mtor, mechanistic target of Rapamycin, is a key regulator of metabolism, cell growth, survival, and proliferation, and also plays an essential role in embryonic growth and development (Land, Scott, & Walker, 2014; Laplante & Sabatini, 2009); Sgk1, regulated by mTOR via phosphorylation and also by Gr through direct transcriptional activation (John et al., 2008), is a serum glucocorticoid kinase that up-regulates ion channels in Xenopus sp. oocytes (Lang & Shumilina, 2013), and is involved in cell survival and postembryonic control of development in Caenorhabditis elegans (Jones, Greer, Pearce, & Ashrafi, 2009); Rictor shares a pathway with Sgk1 and is involved in regulation of fat storage, size and development in C. elegans (Jones et al., 2009); Ghr1 is a growth hormone receptor that is transcribed in very early embryonic stages and suggested to be an important developmental growth factor in vertebrates (Pierce, Breves, Moriyama, Uchida, & Grau, 2012; Sanders &

Harvey, 2004). The six target genes involved in promoting skeletogenesis (Timp2, Ets2, Sparc, Ctsk, Mmp2, and Mmp9, see Table S2) were selected based on their differential expression in divergent Arctic charr morphs from Lake Thingvallavatn, Iceland (Ahi et al. 2014). These extra-cellular matrix (ECM) remodeling genes are essential for skeletogenesis (with roles in trophic skeletogenesis) and make up a highly conserved co-expression network across vertebrates, regulated by transcription factors such as Ets2 (Ahi, 2016).

2.4 | qPCR and normalization

Primers (Table S2) were designed using an assembled Arctic charr transcriptome (Guðbrandsson et al., 2018) and exon boundaries mapped to Salmo salar orthologs from the salmonid species database (Di Genova et al., 2011). Primers spanned at least one exon boundary and were selected based upon their short amplicon size (< 250 bp). Primer efficiencies were calculated (Table S2) and both RT-qPCR and differential mRNA gene expression calculations for each target gene performed according to Ahi et al. (2014), using the reference genes Actb and Ef1a. These reference genes were normalized by randomly selecting one individual within each developmental stage as a calibrator sample. Relative expression quantities (RQ) were calculated according to Ahi et al. (2014).

2.5 | Statistical analyses

All statistical analyses of gene expression were conducted in R version 3.3.2, (R Core Team, 2016) on $\log_2$ transformed RQ values. Normality was investigated by examining the residuals of each model using QQ plots and histograms. To control for non-independence of data from a given family and cage, both female identity and cage were included in all models as a random intercept. Cage was not nested within female since some females had small clutches and therefore only a single cage. Finally, Pearson's correlation coefficients were calculated per developmental stage to determine the extent to which genes may be co-expressed (see Fig. S1).

2.6 | Developmental stage effects

We initially ran a model to test for effects of developmental stage, gene identity and their interaction (individual size was not included in this model as size is confounded with developmental stage) on $\log_{(2)}$ transformed relative mRNA expression values (note, however, that any differences in gene expression at FF compared to other developmental stages may in part reflect sampling procedures as RNA was extracted from the whole embryo at PF, E, and H, but only from the head at FF stage). Due to significant interactions between developmental stage and gene identity (see Table S3), subsequent analyses were conducted within each gene separately using linear mixed effects models (LME) by fitting the lmer function from the lme4 package (Bates, Mächler, Bolker, & Walker, 2015). In these models, developmental stage was used as a fixed effect, female and cage as random effects. Post-hoc Tukey adjusted population Least square means (LS means) and pairwise comparisons were calculated for developmental stage effects using the functions lsmeansLT and difflsmeans, respectively, from the lsmeans package (Lenth, 2016).

2.7 | Size correlated gene expression

To test for dependency of gene expression on individual size, a LME model was fitted per developmental stage. These models included gene identity (14 levels) as a fixed factor (as above) and individual size, as well as its interaction with gene identity, as a covariate. In all models, female identity and cage were included as random effects in the full model. The anova function was used to determine whether the best fitting model (within a given developmental stage) was linear or polynomial (second or third order) using Akaike Information Criterion (AIC). Subsequent analyses were then conducted for the best fitting model, whereby (for both linear and non-linear models) if the size × gene identity interaction was significant, the slope of gene expression and individual size was compared across all genes to determine how expression of genes was correlated with size using the emtrends "pairs" function from the estimated marginal means (emmeans) package (Lenth, 2017). The test function was then used to determine which slopes were significantly different from zero.

3 | RESULTS

Average egg size per female ($N = 7$) was 4.3 ± 0.09 mm at PF, 4.3 ± 0.1 mm at E, and average standard length of embryos was 14.7 ± 0.36 mm at H and 17.8 ± 0.48 mm at FF (Table 1).

3.1 | Developmental stage effects on gene expression

There was a significant gene × developmental stage interaction on relative expression levels ($F = 14.03$; Num $DF = 39$; Den $DF = 2094.4$; $p < 0.0001$; Table S3). Least-square means of gene expression differed from zero for all genes and developmental stages (genes: $p < 0.0001$), with the exception of Ets2 and Star, which were not detected at PF (Figure 1). Igf1 expression was detected only in a few individuals at PF ($n = 12$; 31%), almost half of which were offspring of the same female (ID = 31). Gene specific LME analyses (Table 2) revealed that gene expression levels varied strongly among developmental stages for almost all genes (all $p < 0.004$),

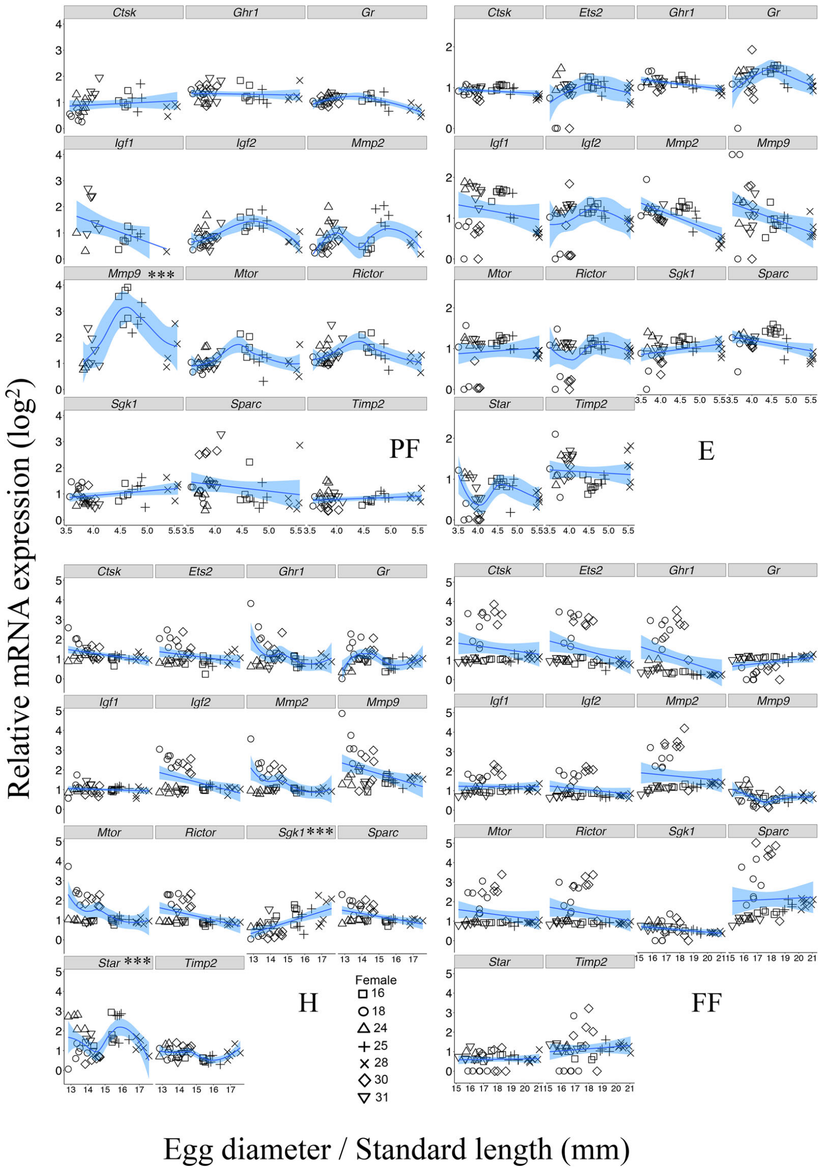

FIGURE 1 Relative gene expression correlations with egg/individual size at post fertilization (PF), eye stage (E), hatching (H) and first feeding (FF) in Arctic charr (Salvelinus alpinus) from lake Vatnshlíðarvatn. Note that at PF, two genes (Star and Ets2) were not detected by qPCR and have been removed from the figure. Both linear and non-linear fits were tested initially for each gene (see Methods section) and the results of the best fitting model represented here. Genes that are significantly correlated with size are Mmp9 at PF, and Sgk1 and Star at H (which best fitted non-linear, linear and non-linear models, respectively, see Table S4). Significance levels are indicated in the gene names of the plots by: p *< 0.01, ** < 0.001 and *** < 0.0001. Symbols correspond to different female identities (i.e., different clutches)

TABLE 2 Linear mixed effects model used to test the effect of developmental stage on gene expression (log expression) in Arctic charr from lake Vatnshlíðarvatn

		Female (Random Effect)		Developmental stage (Fixed Effect)		
Gene identity (growth)	N	X^2	P	Sum Sq	F	P
Star	155	11.10	0.001	45.30	90.52	<0.0001
Igf1	155	8.02	0.005	12.83	19.92	<0.0001
Gr	155	1.16	0.300	1.86	4.57	0.004
Sgk1	155	5.42	0.020	4.14	9.64	<0.0001
Mtor	154	7.94	0.005	4.21	4.74	0.004
Ghr1	154	13.80	0.000	1.44	1.69	0.172
Rictor	154	10.20	0.001	4.88	5.97	<0.001
Igf2	155	5.61	0.020	5.67	8.00	≤0.0001
Gene identity (skeletal)						
Timp2	154	4.32	0.040	4.72	8.90	<0.0001
Sparc	155	11.30	0.001	24.87	18.66	<0.0001
Ctsk	154	3.81	0.050	17.11	18.13	<0.0001
Ets2	155	10.70	0.001	47.78	62.98	<0.0001
Mmp2	154	11.70	0.001	19.45	19.81	<0.0001
Mmp9	155	0.28	0.600	28.94	14.36	<0.0001

N: number of individuals across all developmental stages. χ^2: Chi square statistic. P: P-value (for random effects reflect the likelihood ratio test). Dev.stage: developmental stage. Sum Sq: sum of squares. Den DF: denominator degrees of freedom based on Satterthwaite's approximations. F: F-value. Significance of random (female, $n = 7$) and fixed (developmental stage) effects included for the LME model. Significant results ($P < 0.05$) indicated in bold. Cage was non significant in all cases. Degrees of freedom (DF) of Chi square test and numerator DF were 1 and 3, respectively, in all cases.

apart from Ghr1, which was consistently highly expressed (developmental stage: $p = 0.172$; Table 2 and Figure 2).

Least-square mean expression of three ECM remodeling genes important for skeletogenesis (Ctsk, Ets2, and Mmp2) tended to increase throughout ontogeny, although expression at E stage was not different from PF and H stages (Tukey pairwise $p > 0.05$ for both; Figure 2). For Sgk1, relative expression seemed to decrease throughout ontogeny (Figure 2), however, only the drop at FF was significant. The random effect of family was significant in most cases (Table 2), indicating family level variation in gene expression.

3.2 | Individual size correlated gene expression

Polynomial models of egg size correlated gene expression showed a better fit than a linear model at all developmental stages (see Table S4 for model comparisons and Table 3 for results). Female identity generated much of the variation in gene expression at PF (Figure 1) in a linear model, but this effect was non-significant under a polynomial model (see Table 3). The size × gene identity interaction was significant at all developmental stages (Table 3). This arose in part because relative expression of Mmp9 (at PF), Sgk1, and Star (at H) was correlated with size (all $p < 0.0001$, Table 3). However, when analyzing the effect of size on individual genes using the best-fitting (linear or non-linear) model for each gene separately, Sgk1 had significantly higher expression in larger embryos at H stage (linear model; see Table S4), whereas individual size was non-linearly related to expression of Mmp9 and Star (Figure 1). Post-hoc pairwise comparisons of gene expression (data not shown) revealed that: 1) at PF, Mmp9 had higher expression than all other genes ($p < 0.0001$); 2) at E stage, Timp2 showed lower expression in comparison to Mtor ($t = -3.82$, $p < 0.05$) and Star ($t = -4.08$, $p < 0.01$); 3) at H, Star also had higher expression than all other genes ($p < 0.05$) except Sgk1, which showed significantly higher expression (fitting a non-linear model) than both Rictor and Gr (both $t = 0.62$, $p < 0.05$); and finally, 4) no differences in expression levels were found at FF. When examining gene co-expression across all developmental stages (Figure S1), we find very few weakly co-expressed at PF, which increases at E stage where more growth-related genes are co-expressed than genes involved in skeletogenesis. At H stage, growth related genes such as Star and Sgk1 have a weak positive correlation – but are negatively correlated with all other genes (similar to findings from Ahi et al., 2014), whilst most genes associated with skeletogenesis showed very strong co-expression. This pattern somewhat remains at FF, where the main differences is the drop in co-expression of Mmp9. Throughout all developmental stages, Timp2 does not show any strong correlations with any of the genes used in this study (Figure S1).

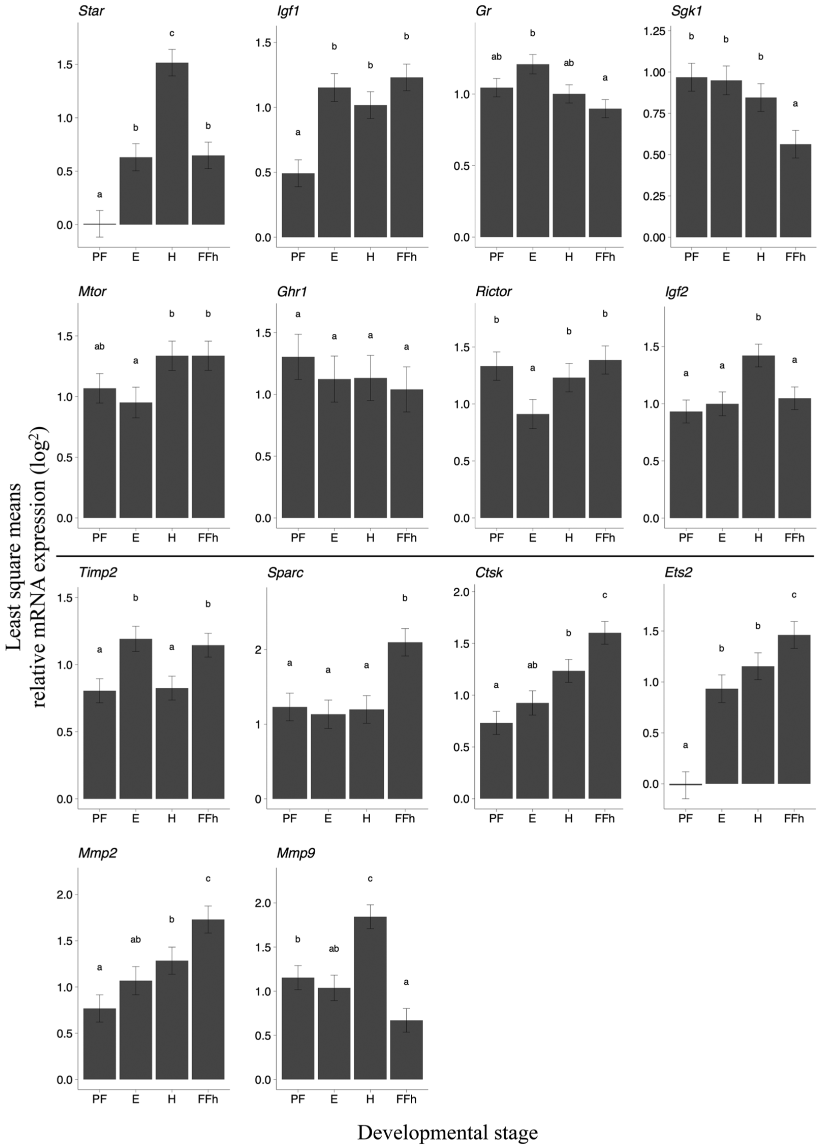

FIGURE 2 Least square (LS) means ± S.E. of relative mRNA expression at four developmental stages: post fertilization (PF), eye stage (E), hatching (H) and first feeding (FF) in Arctic charr (Salvelinus alpinus) from lake Vatnshlíðarvatn. All LS means are significantly different from zero, with the exception of Star and Ets2 at PF stage (not expressed). Least-square means sharing a letter are not significantly different from each other (Tukey-adjusted pairwise comparisons of LS means). Above the centerline are growth related genes, and below are genes involved in skeletogenesis

4 | DISCUSSION

Out of the 14 genes related to growth and/or skeletal development that we analyzed, transcripts of 12 genes were present already at post fertilization (i.e., before the mid-blastula transition, MBT). Expression of only three of the genes correlated with offspring size: Mmp9 at post fertilization, and Sgk1 and Star at hatching stage. Moreover, Sgk1 (a growth-related gene) showed higher expression in larger offspring, whilst expression of Mmp9 and Star was non-linear in relation to offspring size. These findings are somewhat counter to our predictions (with the exception of Sgk1; see below) but do suggest that egg size-correlated expression of genes crucial for skeletogenesis and growth may influence plasticity during early development.

4.1 | Evidence for trophic development

The diversification of trophic structures in teleost fishes has not only resulted in morphologies that match feeding niches (Skúlason & Smith, 1995), but have also facilitated adaptive radiations (Muschick, Indermaur, & Salzburger, 2012). Numerous molecular processes are involved in the development of the vertebrate feeding apparatus (Ahi, 2016), some of which have been involved in the diversification of sympatric morphs of Arctic charr (Ahi et al., 2014). For example, Mmp9, Mmp2, Ctsk, Timp2, and Sparc are all part of the same conserved co-expression network involved in bone remodeling, with Ets2 identified as a potential regulator (Ahi et al., 2014). These genes also seem to be showing similar expression patterns in our study at hatching stage, particularly the strong negative correlated expression of both Star and Sgk1 with all other genes (Figure S1c). At hatching, we also find a large increase in the expression of Mmp9. The glucocorticoid pathway is involved in the regulation of both Mmp9 and Mmp2 during craniofacial skeletogenesis, altering morphogenesis of the pharyngeal cartilages (Hillegass, Villano, Cooper, & White, 2008). Mmp9 transcripts have been reported in pharynx cells in zebrafish (Danio rerio) embryos from 2-5dpf (Sharif, de Bakker, & Richardson, 2014) and osteogenesis (i.e., transformation of cartilaginous skeleton transforms into calcified tissue) is known to occur by hatching (Du, Frenkel, Kindschi, & Zohar, 2001). The involvement of this gene in the development of skeletal elements in the pharyngeal jaw of zebrafish also suggests its likely role in trophic development in Arctic charr (Ahi et al., 2014).

By the critical first feeding stage, we find the gradual increase in expression of Ctsk in the head to be at the highest. Ctsk has a similar role to Mmp9 in osteogenesis and is important for both calcification (Du et al., 2001) and bone remodeling (Petrey et al., 2012). Thus strongly suggesting the potential for this gene to facilitate plasticity of the trophic apparatus in response to diet (e.g., Parsons et al., 2011), especially during first feeding (Figure 2). A gradual increase in expression throughout development was also reflected by Mmp2 (Figure 2), indicating an increased need for ECM remodeling and tissue morphogenesis, such as needed for muscle growth and swimming physiology (Michelin et al., 2009), all of which are crucial for survival after hatching. Three genes involved in bone remodeling (Ets2, Sparc, and Timp2) also increased at the onset of active feeding (Lima, Andrade, Pini, Makrakis, & Makrakis, 2017). It is worth noting the higher expression of genes involved in skeletogenesis may reflect previous findings suggesting that benthivoros charr embryos tend to allocate more energy toward bone development than do pelagic planktivorous charr embryos (Eiríksson et al., 1999). Although direct comparisons of gene expression between the two sympatric morphs found in lake Vatnshlíðarvatn are needed, our current results do demonstrate that the more benthic "brown" Arctic charr morph has – overall – relatively high expression levels of genes related to skeletogenesis throughout ontogeny in comparison to those genes related to growth. The role of these genes in the early development of morphological variation, their sensitivity to different diets (Parsons et al., 2011) and association with egg and embryo size, clearly warrants further attention.

4.2 | Other relevant patterns of gene expression

The transition of embryos from the egg to the external environment requires important physiological changes. One such change that occurs is that of increased larval cortisol, which is essential for an individuals' ability to cope with stress (Mommsen, Vijayan, & Moon, 1999). Our study here, as well as studies on zebrafish, show mRNA levels of Star to be up-regulated immediately before a dramatic rise in basal larval cortisol after hatching (Alsop & Vijayan, 2009). Such elevation may indicate stress induced by hatching. Indeed, evidence from both Rainbow trout (Oncorhynchus mykiss) and zebrafish (Barry, Malison, Held, & Parrish, 1995) suggest that de novo synthesis of this steroid occurs around hatching and, based on our results, this also seems to be the case in Arctic charr.

4.3 | Maternal influence on gene expression at post fertilization

Maternal influence on embryonic development persists until the switch from maternal to embryonic control of gene expression, which occurs at the MBT at 7 days post fertilization (henceforth dpf; 42 DD) in Atlantic salmon (Salmo salar; Nagasawa et al., 2013). Our post fertilization samples were collected 4 dpf (27 DD) and hence prior to the MBT in salmonids. Nevertheless, there is evidence from other studies that zygotic transcripts of genes that are not maternally expressed, can be detected prior to the expected activation of the embryonic genome, that is, for the clearance of maternal mRNAs (Lund, Liu, Hartley, Sheets, & Dahlberg, 2009; Yang, 2002; Zhang, 2003). In the literature on other taxa, we find evidence for pre-MBT transcriptional activity of all genes used in this study, with the exception of Sparc. In our study, the expression of Sparc occurred before the MBT, however, studies on Xenopus do not detect this gene until late gastrulation (Damjanovski, Huynh, Motamed, Sage, & Ringuette, 1998), we therefore suggest that expression of Sparc at post fertilization, may be a product of zygotic

TABLE 3 Results of non-linear mixed effect models testing for the effect of gene identity (fixed factor) and individual size (covariate), as well as their interaction, on gene expression (see Table S5 for results using linear mixed effects models) in Arctic charr from lake Vatnshlíðarvatn

Dev. stage	N	Response	Variable	Variance	SD	X^2	P	Variable	Sum Sq	Num DF	Den DF	F	P	β	SE	DF	t	P
PF	546	Log Expression	Female	0.014	0.12	2.66	0.103	Size (poly2)	1.85	2	6	4.56	0.064					
			Cage	0.001	0.04	0.35	0.556	Genes	94.22	13	489	35.71	<0.0001					
								Size*Genes	38.31	26	489	7.26	<0.0001					
								Mmp9						2.16	0.191	114	11.31	<0.0001
E	485		Female	0.05	0.22	5.97	0.015	Size (poly3)	1.4	3	14	5.23	0.013					
			Cage	0.008	0.09	4.13	0.042	Genes	10.71	13	410	9.25	<0.0001					
								Size*Genes	11.16	39	410	3.21	<0.0001					
H	559		Female	0.07	0.26	8.13	0.004	Size (poly3)	0.22	3	85	0.37	0.778					
			Cage	0.009	0.09	6.58	0.01	Genes	37.73	13	488	14.27	<0.0001					
								Size*Genes	29.76	39	488	3.75	<0.0001					
								Star						0.69	0.137	432	5.02	<0.0001
FF	574		Female	0.185	0.43	15.09	<0.001	Size (poly2)	0.73	2	76	1.06	0.353					
			Cage	0.006	0.08	1.54	0.215	Genes	106.78	13	520	23.81	<0.0001					
								Size*Genes	27.3	26	520	3.04	<0.0001					

Dev.stage; developmental stage. N; total number of observations across all individuals and genes. Log expression; log2 (relative mRNA expression + 1). X^2; Chi square statistic. Chi. DF; number of degrees of freedom for the test. P; P-value of the likelihood ratio test for the random effect. Sum Sq; sum of squares. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Satterthwaite's approximations. F; F-value. Gene slopes that remained significantly different from zero after Tukey's adjustments are indicated, as well as slope (β), standard error (SE), degrees of freedom (DF), t-ratio (t) and associated P-values. Significant variables in bold. PF; post fertlization. E; eye stage. H; hatching stage. FF; first feeding stage. These analyses were conducted separately for each developmental stage due to significant differences among developmental stages (see Table S3). Significance of random (female and cage) and fixed effects are included for the overall models.

transcription. Out of the 14 genes analysed in our study, transcripts of Ets2 and Star were not detectable at the post fertilization stage, strongly suggesting that transcripts of these two genes are not deposited in the egg, but are instead produced by the embryonic genome later in development. In accordance, Star was not expressed until the eye stage in zebrafish (Alsop & Vijayan, 2008). Furthermore, down-regulation of Star occurs in response to excess cortisol (Nesan & Vijayan, 2016), the primary circulating glucocorticoid in fish, whereby the glucocorticoid receptor (Gr) can modulate transcriptional activity of transcription factors, such as Ets2 (Ahi, 2016; Hill, Sussan, Reeves, & Richtsmeier, 2009), which was also found to be undetectable in our study at post fertilization.

4.4 | Evidence for the role of maternal effects

We provide some evidence for egg/offspring size being correlated with gene expression (Mmp9, Star, and Sgk1), but two out of three genes (Mmp9 and Star) contrasted with our original prediction of larger offspring expressing more growth related genes, whilst smaller offspring more skeletal development genes. Previous studies found Sgk1 expression levels to be lower during craniofacial skeletal development in benthic compared to pelagic Arctic charr (Ahi et al., 2014). Here we find lower expression of Sgk1 in smaller offspring (i.e., originating from smaller eggs), which – considering the differential expression of this gene in benthic/pelagic Arctic charr morphs (Ahi et al., 2014) – may be especially relevant in the context of this morph being at the very early stages of divergence. Such changes in gene expression are reflected by changes in the phenotype in several taxa (e.g., Abzhanov et al., 2006; Chan et al., 2010; Uebbing et al., 2016). For instance, previous studies found differential gene expression between sympatric pairs of fish that are often associated with differences along a benthic/pelagic axis (Ahi et al., 2014; Fudickar et al., 2016; Guðbrandsson et al., 2018). Furthermore, differences in ossification and growth rates have been found in sympatric Dolly Varden (Salvelinus malma: Esin, Markevich, & Pichugin, 2018), concomitant to variation in expression of genes involved in the regulation of growth and skeletogenesis (our study). Thus highlighting the need for targeted studies that explicitly link development and growth with underlying patterns of gene expression. Although we find evidence for the role of maternal effects (egg size) in influencing the expression of three genes involved in growth and skeletogenesis, further evidence is clearly needed to make general predictions regarding egg size correlated gene expression.

5 | CONCLUSION

Our results provide evidence for dynamic variation in expression of important genes related to growth and skeletogenesis across early life-stages. We also find some evidence for this variation being correlated with offspring size (for Mmp9 at post fertilization, and Sgk1 and Star at hatching). Interestingly, all three of these genes are differentially expressed in benthic and pelagic Arctic charr morphs (Ahi et al., 2014), suggesting potential for diversification of different phenotypes during early life-stages.

Better understanding the causes of phenotypic divergence at early life-stages and the relationship between egg size and offspring phenotype and fitness – especially under differing dietary/ecological conditions – would shed light both on early stages of divergence along the speciation continuum (e.g., Nosil, 2012) as well as the evolution of optimal maternal investment in contrasting environments (e.g., Smith & Fretwell, 1974). The number of studies documenting egg size mediated effects on offspring phenotype in fishes is rapidly growing (Cogliati et al., 2018; Leblanc et al., 2011; Leblanc et al., 2016; Segers et al., 2012; Self, Schreck, Cogliati, Billman, & Noakes, 2018; Thorn & Morbey, 2018). At the same time, our knowledge as to whether egg size can influence evolutionary divergence is limited. Future studies should address this knowledge gap by combining data on offspring gene expression and phenotypes (e.g., morphology and behavior) across developmental stages in multiple populations along the diversification continuum (i.e., incorporating populations both at early stages of divergence and well-diverged populations). For instance, egg size variation along such a continuum may decline with increasing diversification due to canalization (Parsons et al., 2011; Waddington, 1942), and strong divergence in egg size is also likely in populations inhabiting contrasting environments. Environmental heterogeneity can influence egg size (Koops, Hutchings, & Adams, 2003), and could therefore be considered a factor in the generation of different phenotypes especially given the potential for egg size mediated differences in gene expression (Segers et al., 2012), as well as phenotype (e.g., Cogliati et al., 2018). In general, our findings on gene expression suggest that maternal effects and early developmental changes in gene expression may influence developmental plasticity, yet studies combining both gene expression and phenotypic variation are needed to further our understanding on the mechanisms of early phenotypic divergence.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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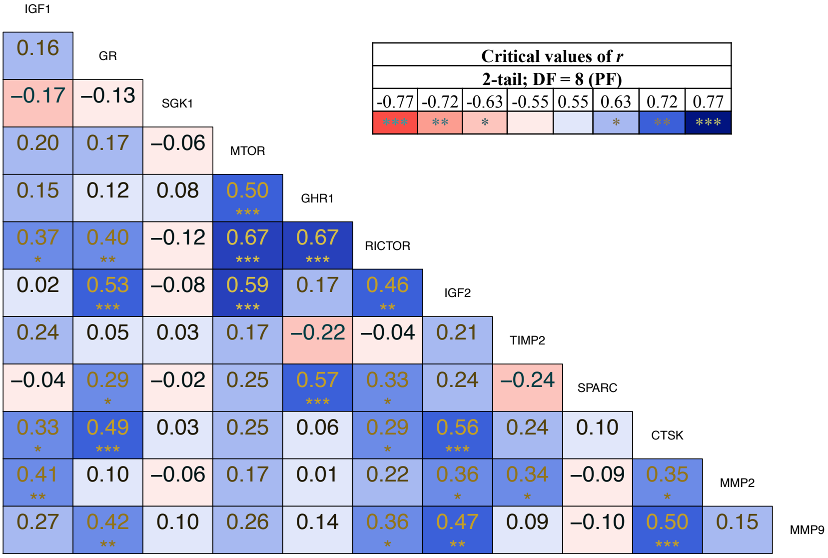
3.1 Appendix

Figure S3.1 *Pearsons' correlation coefficients between expression levels of 14 genes related to growth (Star/Igf1 – Igf2) and skeletogenesis (Timp2 – Mmp9), across four developmental stages in the brown morph of Arctic charr from lake Vatnshlíðarvatn: a) post-fertilisation (PF), b) eye stage (E), c) hatching (H), and d) first feeding (FF). Note that at PF, two genes were not detected by qPCR (Star and Ets2) and panels at this developmental stage are therefore in a different order in comparison to E, H and FF.*

Post-fertilisation

a)

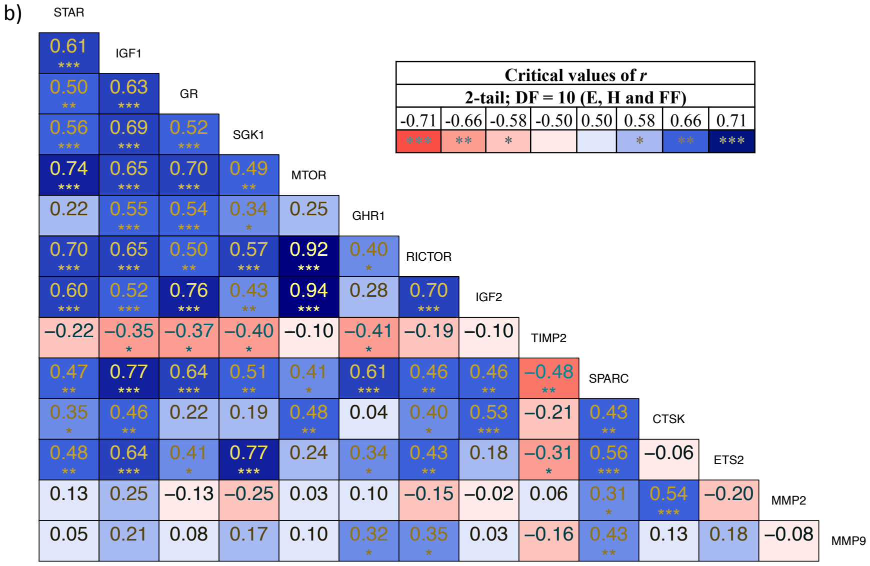


	IGF1	GR	SGK1	MTOR	GHR1	RICTOR	IGF2	TIMP2	SPARC	CTSK	MMP2
GR	0.16										
SGK1	−0.17	−0.13									
MTOR	0.20	0.17	−0.06								
GHR1	0.15	0.12	0.08	0.50 ***							
RICTOR	0.37 *	0.40 **	−0.12	0.67 ***	0.67 ***						
IGF2	0.02	0.53 ***	−0.08	0.59 ***	0.17	0.46 **					
TIMP2	0.24	0.05	0.03	0.17	−0.22	−0.04	0.21				
SPARC	−0.04	0.29 *	−0.02	0.25	0.57 ***	0.33 *	0.24	−0.24			
CTSK	0.33 *	0.49 ***	0.03	0.25	0.06	0.29 *	0.56 ***	0.24	0.10		
MMP2	0.41 **	0.10	−0.06	0.17	0.01	0.22	0.36 *	0.34 *	−0.09	0.35 *	
MMP9	0.27	0.42 **	0.10	0.26	0.14	0.36 *	0.47 **	0.09	−0.10	0.50 ***	0.15

Critical values of *r* 2-tail; DF = 8 (PF)							
-0.77	-0.72	-0.63	-0.55	0.55	0.63	0.72	0.77
***	**	*			*	**	***

Eye stage

b)

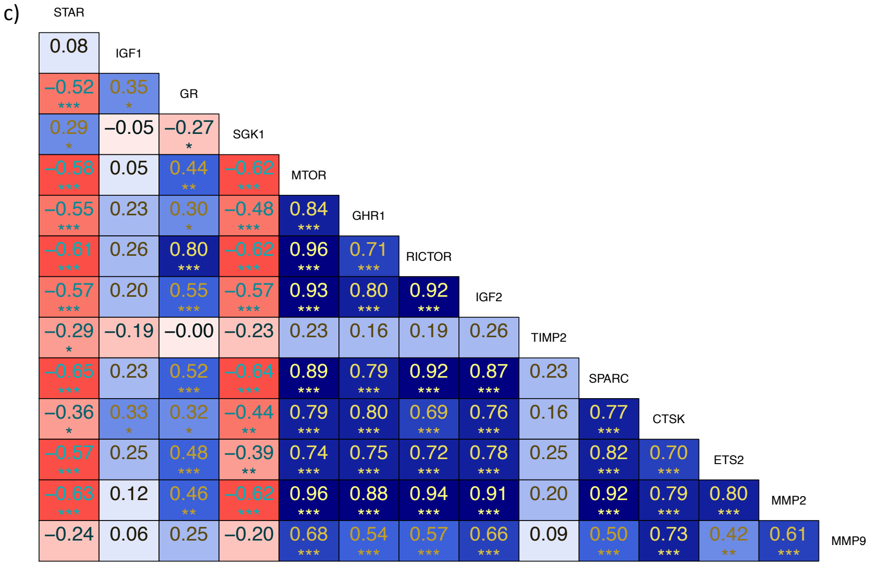


	STAR	IGF1	GR	SGK1	MTOR	GHR1	RICTOR	IGF2	TIMP2	SPARC	CTSK	ETS2	MMP2
IGF1	0.61 ***												
GR	0.50 **	0.63 ***											
SGK1	0.56 ***	0.69 ***	0.52 ***										
MTOR	0.74 ***	0.65 ***	0.70 ***	0.49 **									
GHR1	0.22	0.55 ***	0.54 ***	0.34 *	0.25								
RICTOR	0.70 ***	0.65 ***	0.50 **	0.57 ***	0.92 ***	0.40 *							
IGF2	0.60 ***	0.52 ***	0.76 ***	0.43 **	0.94 ***	0.28	0.70 ***						
TIMP2	−0.22	−0.35 *	−0.37 *	−0.40 *	−0.10	−0.41 *	−0.19	−0.10					
SPARC	0.47 **	0.77 ***	0.64 ***	0.51 **	0.41 *	0.61 ***	0.46 **	0.46 **	−0.48 **				
CTSK	0.35 *	0.46 **	0.22	0.19	0.48 **	0.04	0.40 *	0.53 ***	−0.21	0.43 **			
ETS2	0.48 **	0.64 ***	0.41 *	0.77 ***	0.24	0.34 *	0.43 **	0.18	−0.31 *	0.56 ***	−0.06		
MMP2	0.13	0.25	−0.13	−0.25	0.03	0.10	−0.15	−0.02	0.06	0.31 *	0.54 ***	−0.20	
MMP9	0.05	0.21	0.08	0.17	0.10	0.32 *	0.35 *	0.03	−0.16	0.43 **	0.13	0.18	−0.08

Critical values of *r*							
2-tail; DF = 10 (E, H and FF)							
-0.71	-0.66	-0.58	-0.50	0.50	0.58	0.66	0.71
***	**	*			*	**	***

Hatching

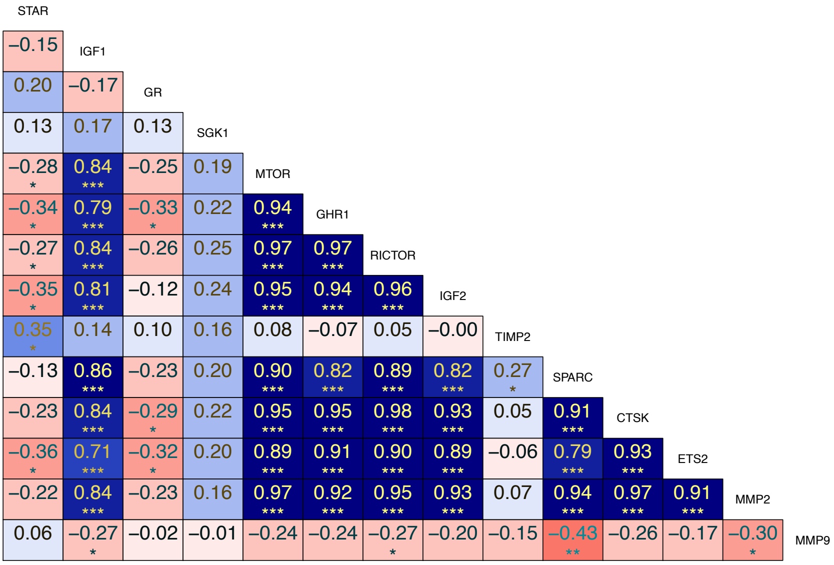
c)



	STAR	IGF1	GR	SGK1	MTOR	GHR1	RICTOR	IGF2	TIMP2	SPARC	CTSK	ETS2	MMP2
IGF1	0.08												
GR	−0.52 ***	0.35 *											
SGK1	0.29 *	−0.05	−0.27 *										
MTOR	−0.58 ***	0.05	0.44 **	−0.62 ***									
GHR1	−0.55 ***	0.23	0.30 *	−0.48 ***	0.84 ***								
RICTOR	−0.61 ***	0.26	0.80 ***	−0.62 ***	0.96 ***	0.71 ***							
IGF2	−0.57 ***	0.20	0.55 ***	−0.57 ***	0.93 ***	0.80 ***	0.92 ***						
TIMP2	−0.29 *	−0.19	−0.00	−0.23	0.23	0.16	0.19	0.26					
SPARC	−0.65 ***	0.23	0.52 ***	−0.64 ***	0.89 ***	0.79 ***	0.92 ***	0.87 ***	0.23				
CTSK	−0.36 *	0.33 *	0.32 *	−0.44 **	0.79 ***	0.80 ***	0.69 ***	0.76 ***	0.16	0.77 ***			
ETS2	−0.57 ***	0.25	0.48 ***	−0.39 **	0.74 ***	0.75 ***	0.72 ***	0.78 ***	0.25	0.82 ***	0.70 ***		
MMP2	−0.63 ***	0.12	0.46 **	−0.62 ***	0.96 ***	0.88 ***	0.94 ***	0.91 ***	0.20	0.92 ***	0.79 ***	0.80 ***	
MMP9	−0.24	0.06	0.25	−0.20	0.68 ***	0.54 ***	0.57 ***	0.66 ***	0.09	0.50 ***	0.73 ***	0.42 **	0.61 ***

First-feeding

d)



	STAR	IGF1	GR	SGK1	MTOR	GHR1	RICTOR	IGF2	TIMP2	SPARC	CTSK	ETS2	MMP2
IGF1	−0.15												
GR	0.20	−0.17											
SGK1	0.13	0.17	0.13										
MTOR	−0.28 *	0.84 ***	−0.25	0.19									
GHR1	−0.34 *	0.79 ***	−0.33 *	0.22	0.94 ***								
RICTOR	−0.27 *	0.84 ***	−0.26	0.25	0.97 ***	0.97 ***							
IGF2	−0.35 *	0.81 ***	−0.12	0.24	0.95 ***	0.94 ***	0.96 ***						
TIMP2	0.35 *	0.14	0.10	0.16	0.08	−0.07	0.05	−0.00					
SPARC	−0.13	0.86 ***	−0.23	0.20	0.90 ***	0.82 ***	0.89 ***	0.82 ***	0.27 *				
CTSK	−0.23	0.84 ***	−0.29 *	0.22	0.95 ***	0.95 ***	0.98 ***	0.93 ***	0.05	0.91 ***			
ETS2	−0.36 *	0.71 ***	−0.32 *	0.20	0.89 ***	0.91 ***	0.90 ***	0.89 ***	−0.06	0.79 ***	0.93 ***		
MMP2	−0.22	0.84 ***	−0.23	0.16	0.97 ***	0.92 ***	0.95 ***	0.93 ***	0.07	0.94 ***	0.97 ***	0.91 ***	
MMP9	0.06	−0.27 *	−0.02	−0.01	−0.24	−0.24	−0.27 *	−0.20	−0.15	−0.43 **	−0.26	−0.17	−0.30 *

Numbers indicate Pearson’s pairwise correlation coefficients between expression levels of seven females of Arctic charr at each developmental stage. Blue represents positive and red represents negative expression correlation. A scale showing critical values of *r* is depicted with corresponding colours. Critical values of *r* for PF is different to the other developmental stages as two genes (*Star* and *Ets2*) were not detected by qPCR. DF, degrees of freedom. P: *< 0.01, **<0.001 and ***<0.0001.

Table S3.1 *Additional female characteristics that were measured for fork length and offspring size, but not used for gene expression analysis, are shown across four developmental stages (1) post-fertilisation, PF; 2) eye stage, E; 3) hatching, H; and 4) first feeding, FF) in the brown morph of Arctic charr from lake Vatnshlíðarvatn.*

Female					**Offspring stage**															
					PF				**E**				**H**				**FF**			
Female identity	**Year**	**Fork length (cm)**	**Weight after (g)**	**Weight before (g)**	**Mean egg diameter (mm)**	**SD**	**DD**	**N**	**Mean egg diameter (mm)**	**SD**	**DD**	**N**	**SL (mm)**	**SD**	**DD**	**N**	**SL (mm)**	**SD**	**DD**	**N**
17	2014	17	46		5.1	0.08	27	6	5.1	0.17	242	6	16.5	0.07	473	6	20.2	0.87	701	10
23	2014		45		3.6	0.1	27	6	3.6	0.11	242	6	13	0.49	462	6	15.1	0.28	701	10
26	2014	13.7	27.5	30	3.6	0.05	27	6	3.7	0.07	242	6	12.7	0.59	473	6	14.9	1	701	8
135	2015	17.6	60	74	4.4	0.15	12	7	4.5	0.12	217	12	15.2	0.43	470	9	18.4	0.61	639	13
140	2015	18	65		4.4	0.12	12	8	4.4	0.08	230	5	15	0.37	442	7	18.5	0.36	639	15
143	2015	24.8	131	146	4.4	0.13	12	16	4.4	0.1	217	14	13.6	0.24	386	14	17.5	2.63	639	12
149	2015	17.2	54		4.4	0.08	12	7	4.4	0.13	230	6	14.3	0.33	440	15	17.5	0.44	639	5
150	2015	24.1	128	160	5.4	0.12	12	10	5.3	0.15	217	14	14.5	1.55	419	17	19.9	0.49	639	12
152	2015	15.7	44		4.2	0.1	12	8	4.4	0.09	217	6	14.3	0.45	442	7	16.5	0.6	639	9
154	2015	19.8	76	84	4.2	0.09	12	8	4.3	0.08	217	5	14.5	0.34	438	6	17.3	0.49	610	8

Mean individual size (egg diameter, mm) or (SL, standard length) of offspring (N) with associated standard deviation (SD) were measured for all females. Sample sizes (N) represent all individuals that have been preserved in an RNA stabilising buffer and measured. Blank spaces indicate missing data. Female weight before and after stripping eggs is given when available.

Table S3.2 *Primer sequences and relevant information for Arctic charr (Salvelinus alpinus).*

Gene	Description	Primer Sequence (5’- 3’)	Product Size (bp)	PCR Efficiency	Exon boundary	Accession no.
Actb	Beta Cytoskeletal Actin	F-GAAGATCAAGATCATCGCCC R-CAGACTCGTCGTACTCCTGCT	122	1.95	Yes	JR540730
Ctsk	Cathepsin K	F-CAGATGAGGCTTACCCTTACCTTGG R-ACACTGAGCACCCATGCCAG	74	1.98	Yes	LM643986
Ets2	V-Ets Erythroblastosis Virus E26 Oncogene Homolog 2 (Avian)	F-AAGACTACCCTGCTGACC R-CTCTCCAATGAACACAAACTCTG	155	1.92	Yes	LM644000
Mmp2	Matrix Metallopeptidase 2	F-ATGGATGGAGAGGCTGACATC R-GGTCCAGGAGCAAAGGCAT	110	1.94	Yes	KC538875.1
Mmp9	Matrix Metallopeptidase 9	F-AATCTACTTCTTCTCAGGCACC R-CTCCCTCCACCTTCTCTAC	111	1.9	Yes	LM643995
Gr	Glucocorticoid Receptor	F-CGCTCCTACCAGCAGTGTA R-GGCAACTTCATCCTCTCATCAT	80	1.96	Yes	LM643989
Sgk1	Serum/Glucocorticoid Regulated Kinase	F-CCTCCGTTCTACAGTCGTAA	113	1.88	Yes	LM644002

	1	R-AGCAGTCCCTCCAGTATGT				
Sparc	Secreted Protein, Acidic, Cysteine-Rich (Osteonectin)	F-GTTCTGGTCACCCTGTACGAG R-GCAGTCTCTTCTCATTCTCATAGATC	100	1.92	No	KC538874.1
Star	Steroidogenic Acute Regulator	F-TCTGCTTGTGTAATCATAGCCC R-GTGGGAATGTGTGTATGTTTTGAG	104	1.92	Yes	AF232215.1
Timp2	Tissue Inhibitor of Metalloproteinase 2	F-GTCATACTTGATCTCGTTAGACAAA R-CAACAGGCTTTTTGCGATG	84	1.94	Yes	LM644001
Igf2	Insulin Like Growth Factor 2	F-GGAGACGCTATGTGGAGGAGAACT R-TGCTGTTAGACCTGCTGGTTGG	98	1.9	Yes	GU933430.1
Ghr1	Growth Hormone Receptor 1	F-ACATCCCCACTAAAGAGTCCCGA R-GGAGAATCACCATCAACTTCTGTTGC	123	1.98	Yes	XM_014133894.1
Mtor	Mechanistic Target Of Rapamycin Kinase	F-GGCACAGGTTCTCGTGCGA R-GAGACAAGCTGACTGGGCGTGA	105	1.89	Yes	XM_024135341.1
Rictor	Rapamycin-Insensitive Companion Of MTOR	F-GATCCACCCGTCTCTGTCTCATAGT R-GCTCAACGCCTTCAGAAACCTG	136	1.93	Yes	XM_024001547.1

Table S3.3 *A mixed effects model to test for the effects of developmental stage (dev. stage), gene identity (gene) and their interaction on gene expression in Arctic charr from lake Vatnshlíðarvatn. The model included female identity (N= 7) and cage as random effects to account for non-independence of data.*

	SS	**MS**	**NumDF**	**DenDF**	**F**	***P***
Dev.stage	51.38	17.1	3	1900	53.33	**<0.0001**
Gene	70.74	5.4	13	2094	16.94	**<0.0001**
Dev.stage x Gene	175.72	4.5	39	2094	14.03	**<0.0001**

Sum Sq; sum of squares. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Satterthwaite's approximations. F = F-value. *P* =*P*-value to determine the significance of these fixed effects on relative gene expression (log^2).

Table S3.4 *Model selection results using the Akaike Information Criterion (AIC) to determine whether gene identity (fixed factor), individual size (covariate), as well as their interaction on gene expression was best fitted using a linear or non-linear (polynomial) model across four developmental stages of Arctic charr from lake Vatnshlíðarvatn: PF, post-fertilisation; E, eye stage; H, hatching; and FF, first feeding. The model included female identity (N = 7) and cage as random effects to account for non-independence of data.*

Dev. stage	**Model**	**Df**	**AIC**	X^2	***X* Df**	***P***
PF	Linear	31	824			
	Polynomial^2	45	740	111.91	14	**<0.0001**
	Polynomial^3	59	743	24.36	14	0.041
E	Linear	31	325			
	Polynomial^2	45	304	49.40	14	<0.0001
	Polynomial^3	**59**	**297**	**34.80**	**14**	**0.002**
H	Linear	31	793			
	Polynomial^2	45	803	17.71	14	0.2206
	Polynomial^3	59	787	44.12	14	**<0.0001**
FF	Linear	31	1116			
	Polynomial^2	45	1095	48.89	14	**<0.0001**
	Polynomial^3	59	1096	27.01	14	0.019

Dev.stage, developmental stage. Df, degrees of freedom. AIC, Akaike Information Criterion. X^2, chi square.

Table S3.5 *Results of linear mixed effect models testing for the effect of gene identity (fixed factor) and individual size (covariate), as well as their interaction, on gene expression in Arctic charr from lake Vatnshlíðarvatn. These analyses were conducted separately for each developmental stage due to significant differences among developmental stages (see Table S3.3). Significance of random (female and cage) and fixed effects are included for the overall models.*

			Random Effects				Fixed Effects										
Dev. stage	N	Response	Variable	Chi. sq	Chi. DF	*P*	Variable	Sum Sq	Num DF	Den DF	F	P	β	SE	DF	t	*P*
PF	546	Log Expression	**Female**	12.733	1	**<.0001**	Size	0.12	1	8	0.50	0.497					
			Cage	0.048	1	0.827	**Gene**	16.54	13	505	5.29	**<.0001**					
							Size*Gene	16.53	13	505	5.29	**<.0001**					
							Mmp9						1.026	0.174	35	5.882	**<0.0001**
E	485		**Female**	14.600	1	**<.0001**	Size	0.08	1	10	0.75	0.407					
			Cage	0.000	1	1	**Gene**	5.52	13	452	4.16	**<.0001**					
							Size*Gene	4.66	13	452	3.51	**<.0001**					
H	559		**Female**	9.035	1	**0.003**	Size	0.16	1	44	0.75	0.393					
			Cage	6.840	1	**0.009**	**Gene**	20.60	13	516	7.36	**<.0001**					
							Size*Gene	17.88	13	516	6.39	**<.0001**					
							Sgk1						0.339	0.074	182	4.53	**0.0001**
FF	574		**Female**	16.648	1	**<.0001**	Size	0.39	1	69	1.07	0.306					
			Cage	1.115	1	0.291	**Gene**	12.94	13	533	2.72	**0.001**					
							Size*Gene	11.33	13	533	2.38	**0.004**					

Dev.stage; developmental stage. N; total number of observations across all individuals and genes. Log expression; log2 (relative mRNA expression + 1). X^2; Chi square statistic. Chi. DF; number of degrees of freedom for the test. *P*; *P*-value of the likelihood ratio test for the random effect. Sum Sq; sum of squares. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Satterthwaite's approximations. F; F-value. Gene slopes that remained significantly different from zero after Tukey's adjustments are indicated, as well as slope (β), standard error (SE), degrees of freedom (DF), t-ratio (*t*) and associated *P*-values. Significant variables in bold. PF; post-fertlisation. E; eye stage. H; hatching stage. FF; first feeding stage.

4 Paper III

The importance of family effects for craniofacial shape variation at early life-stages in Arctic charr (*Salvelinus alpinus*)

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Authors' contribution: Conceived and designed the experiments: SVB CAL ZOJ BKK KR SS. Performed the experiments: SVB CAL. Analysed the data: SVB. Contributed reagents/materials/analysis tools: SVB CAL KR ZOJ BKK. Wrote the paper: SVB CAL KR BKK SS ZOJ.

4.1 Abstract

Intraspecific variation in traits associated with sympatric divergence may be key to understanding the drivers of evolutionary diversification. The trophic apparatus is amongst these traits that have been found to influence sympatric speciation. However mechanisms underlying this phenotypic variation are little understood. Here we propose that maternal effects, i.e. variation in egg size and therefore offspring size, might provide the initial variation by which the offsprings' developmental trajectory is altered to accommodate for the differential distribution of maternal resources. We use a single morph of the salmonid Arctic charr (*Salvelinus alpinus*) that is believed to be at the very early stages of sympatric divergence, to determine whether offspring craniofacial shape is influenced by individual size. Based on previous findings documenting a more benthic behaviour of offspring from smaller eggs after first feeding, we predict that smaller offspring will have a wider hyoid arch that is more related to a benthic phenotype. We used acid-free double staining combined with geometric morphometrics, and placed 17 fixed landmarks and 54 sliding landmarks in structures associated with the feeding apparatus in the head. We found that individual size has no effect on morphology, but instead demonstrate a strong family effect in offspring at first feeding, but not at hatching. We suggest that being released from the spatial constraints of the egg may initiate differences in developmental trajectories, so that although such differences were not observable at hatching, they become enhanced by the onset of exogenous feeding. This study provides further evidence to the growing documentation of family effects being an important driver in the development of individuals, especially in salmonids. Such variation may even facilitate divergence in resource use, resulting in increased intraspecific phenotypic variation and possibly the evolution of resource polymorphisms.

4.2 Introduction

Individual specialisation refers to when individuals within a population have narrower niches than the population as a whole, for reasons others than those that can be contributed by age, sex, or discrete morphological group (Bolnick et al., 2003). Such individual specialisation could be found to promote the development of increased morphological variation, within populations, through phenotypic plasticity facilitating the exploitation of a particular habitat/resource by some individuals, which may increase their fitness (Forsman, 2014). This phenotypic variation originates during early development, as a response to various genetic and environmental inputs, which may have lasting effects on an individuals' phenotype (West-Eberhard, 2003; Hoverman & Relyea, 2007; Uller, 2008; Nettle & Bateson, 2015). Such phenotypic or developmental plasticity (hereon referred to under the umbrella term 'plasticity'; Uller, 2008) may further facilitate longer-term evolutionary adaptation via genetic accommodation and assimilation (Waddington, 1953; West-Eberhard, 2003; Pfennig et al., 2010). Individual specialisation may therefore have the potential to promote resource polymorphism (i.e. the formation of discrete intraspecific morphs utilising different resources; Smith & Skúlason, 1996; Pfennig & Martin, 2009) and subsequent sympatric speciation (Schluter, 2000; Nosil, 2012).

Maternal effects, the influence of maternal genotype or phenotype on offspring phenotype (e.g. Wolf & Wade, 2009), are an example of plasticity that can span multiple generations (i.e. transgenerational plasticity; Mousseau & Fox, 1998; Uller, 2008). For example, plasticity in maternal investment (i.e. mRNA, miRNA, yolk and hormones deposited in the eggs) in response to environmental conditions has the potential to alter offspring developmental trajectories, resulting in offspring that may be better 'equipped' to cope with novel conditions by being able to adjust aspects of their development (Agrawal, 2001; Uller, 2008; Macagno et al., 2018). For instance, in spadefoot toads (*Spea multiplicata*), differential maternal investment (i.e. egg size) mediates plasticity in offspring traits (such as size), contributing to rapid population divergence and character displacement under increased competition for resources (Pfennig & Martin, 2009; Pfennig et al., 2010). Such variation in egg/offspring size can result in offspring optimising their resources towards different functions (such as growth or bone ossification), which can influence individual specialisation, e.g. if food resources become scarce (Bolnick et al., 2003; Cucherousset et al., 2011).

Despite some understanding of ecological drivers of morphological diversity (Skúlason & Smith, 1995; Kristjánsson et al., 2011), the underlying mechanisms and processes by which plasticity might be able to promote phenotypic innovation and contribute towards population divergence, are less well-understood (Pfennig et al., 2010; Moczek et al., 2011; Donelson et al., 2018; Macagno et al., 2018). Egg size is one such mechanism that has received recent interest in this context (Leblanc et al., 2011, 2016; Ahi et al., 2018; Cogliati et al., 2018; Penney et al., 2018; Self et al., 2018; Thorn & Morbey, 2018). Egg size has been seen to affect phenotypic variation in hatchery-reared Chinook salmon (*Onchorynchus tshawytscha*), with offspring from smaller eggs having deeper bodies than those from larger eggs, which had a more fusiform body shape (Cogliati et al., 2018). In brook trout (*Salvelinus fontinalis*), offspring from larger eggs were more plastic than their smaller conspecifics (Penney et al., 2018). Furthermore, evidence from a study on cichlids showed that mouth-gaping frequency during embryonic development resulted in differential craniofacial development (Hu & Albertson, 2017). It is uncertain whether embryonic movements within the egg, as well as after hatching, can influence craniofacial development. Differences in egg size can be expected to influence the ability of embryos to move within eggs, whilst respiratory movements are more likely to occur at hatching. This mobility could potentially cause differences in the development of skeletal elements.

Here, we aim to understand how variation in maternal provisioning (egg size) can influence offspring trophic morphology, known to be important for diversification in fishes (Hulsey et al., 2005). We test to what extent individual size, which is strongly correlated with egg size (Heath et al., 1999; Leblanc et al., 2016; Cogliati et al., 2018; Penney et al., 2018), influences within morph trophic morphology at early life stages in the salmonid fish Arctic charr (*Salvelinus alpinus*). Arctic charr are famed for their extensive intra-lacustrine resource polymorphism (Smith & Skúlason, 1996) that has repeatedly evolved across lakes (Kapralova et al., 2011), often with multiple morphs that differ in body size, behaviour, life-history traits, and morphologies (Skúlason et al., 1999; Klemetsen, 2010). Such propensity for intraspecific diversity makes this species well suited for studies of the mechanisms that promote and/or precede the evolutionary origins of phenotypic diversity, especially associated with trophic polymorphism.

Several key aspects were of particular relevance for this study. First, phenotypic studies on a large benthic (LB) and a planktivorous (PL) morphs in lake Þingvallavatn, Iceland, found that progeny of these two morphs differ phenotypically at very early developmental stages (Skúlason et al., 1989); for example, with the benthic morph having a wider hyoid arch (a structure known to be important for feeding by expanding the buccal cavity, facilitating prey capture by creating a suction force as the hyoid arch depresses; Hulsey et al., 2005), than the pelagic morph (Kapralova, 2014). Second, maternal investment in egg size can be highly variable among and within Arctic charr females (Wallace & Aasjord, 1984; Baroudy & Elliott, 1994; Benhaïm et al., 2003; Leblanc et al., 2011). Third, variation in egg size influences the phenotype of Arctic charr offspring (Benhaïm et al., 2003; Leblanc et al., 2011, 2016). Larger eggs result in larger offspring that feed mainly in the water column and at the surface in comparison to smaller offspring, which resided and foraged more at the bottom indicating differential resource use shortly after first feeding (Benhaïm et al., 2003; Leblanc et al., 2011, 2016). In laboratory conditions, these size-related differences may also persist up until maturity (Bjorklund et al., 2003). Initially larger individuals of Arctic charr were known to invest more in somatic growth, whilst initially smaller individuals tended to allocate more energy towards bone ossification, likely as a consequence of their small size (Eiríksson et al., 1999; Valdimarsson et al., 2002). Despite their faster bone ossification, which may include trophic structures, our previous study (Beck et al., *Paper I*) did not find that smaller individuals started to feed earlier. Finally, early life-stages show dynamic expression of genes related to skeletogenesis, however, evidence for egg size-related gene expression was limited (Beck et al., 2018 and *Paper IV*).

Here we use the so-called 'brown' Arctic charr morph (VB) from lake Vatnshlíðarvatn, NW Iceland (Jónsson & Skúlason, 2000) as our model to test whether early individual size correlates with trophic morphology. This morph is a good candidate for studies of maternal effects because absolute egg size is highly variable within and among females of similar sizes (personal observations, *Paper I*). Using acid-free double staining of cartilage and bone coupled with geometric morphometrics, we test whether individuals from different sized eggs develop differently, resulting in different trophic morphologies (Waddington, 1942). Specifically, we examined the following hypotheses and predictions: 1) morphology will differ between the two developmental stages (hatching and first feeding), as seen in other early developmental stages in Arctic charr morphs (Kapralova et al., 2015); 2) offspring size will be correlated with morphology, due to variation in maternal investment (i.e. the egg; Leblanc et al., 2016) that provides differently to pathways for growth and development (i.e. mRNA, hormones, yolk); and 3) smaller individuals will have a wider hyoid arch, relating to a more benthic phenotype, whilst larger individuals will have a narrower hyoid arch, relating to a more pelagic phenotype (Benhaïm et al., 2003; Leblanc et al., 2011; Kapralova, 2014), reflecting differences in behaviour at the onset of feeding (Leblanc et al., 2011).

4.3 Materials and methods

4.3.1 Study system

Vatnshlíðarvatn (65°517.630 N, -19°617.688W) is a physically simple, small and shallow lake (Jónsson & Skúlason, 2000). Arctic charr is the only fish species in the lake and has diverged in to two morphs (silver [VS] and brown), with the VB morph being smaller and having a stocky body and light brown body colour in comparison to VS (Jónsson & Skúlason, 2000; Parsons et al., 2011). In VB, egg diameter and standard length of the embryos at hatching and first feeding were strongly correlated (N = 12 families, hatching: $R^2 = 0.96$, N offspring = 201; first feeding: $R^2 = 0.97$, N offspring = 168; both $P < 0.001$; Leblanc et al. unpublished data).

Mature females (N=7) and males (N= 5) were caught using gill nets (15-19 mm mesh size) in early September 2014. Four of the females (ID = 16, 18, 24 and 30) were each mated to a single male (full-sib offspring), whereas three females (ID = 25, 28 and 31) were mated to a single male (paternal half-sibs). Such effects can therefore not be strictly referred to as either ‘family or ‘female’ effects, yet for simplicity we hereon refer to such variation in our sampling design as ‘family’ effects. Although this design causes variation in the relatedness of offspring, it was used to minimise the risk of unsuccessful fertilisation, whilst also reducing potential direct genetic effects. Eggs and milt were collected, mixed in the field, and the fertilised eggs were allowed to water-harden for one hour before transport to Hólar University College’s aquaculture facilities in Verið, Sauðárkrókur.

Embryos were reared in cages (6.5cm x 6.5cm x 5cm) with 2cm x 2cm holes at the front and rear. The holes and the bottom of the cage were covered with mesh (1mm) to allow for good oxygenation and free flowing water. Cages were placed in a shelf incubator system (MariSource 8-tray Vertical Incubator) with a constant 95% recycled water flow. For most families, a single cage was used (one layer of eggs), but in cases where eggs were more numerous they were split into two to three separate cages. Embryos were reared in darkness, to mimic natural conditions, with a mean water temperature of 4.1ºC (SD ± 0.38), recorded by temperature loggers four times daily. Dead individuals (opaque eggs) were manually removed every two days to prevent fungal growth. An accumulative temperature estimate (degree days, DD; Pruess, 1983) was used to determine the approximate time by which developmental stages occurred.

Samples (total N = 95) were randomly collected at two developmental stages: 1) hatching (H, 461 DD, SD ± 7.87, N = 42), when individuals have come out of the egg but still rely on nutrition from the yolk sac; and 2) first feeding (FF, 696 DD, N = 53), when individuals begin feeding (Ballard, 1973; see Table 4.1 for sample summary), and were euthanised using 2-phenoxyethanol (Pounder et al., 2017). Individuals were photographed (Canon EOS 650D) on their left side with a length scale, and standard length (SL; Leblanc et al., 2016) measured using the program Fiji (Schindelin et al., 2012).

4.3.2 Staining and digitisation

The collected individuals were fixed in 4% paraformaldehyde (PFA) and stained red (Alizarin) for bone and blue (Alcian) for cartilage, using a modified protocol from (Walker & Kimmel, 2007; Kapralova, 2014; see Appendix S1). Based on a previous study by Kapralova et al. (2015), as well as preliminary trials, we selected craniofacial elements that were clearly stained and visible at both H and FF stages (see Fig. 4.1). For a comprehensive overview on all structures visible in developing embryos of Arctic charr, as well as the timing of appearances of each craniofacial cartilage/bone considered in this study, see Kapralova et al. (2015).

Table 4.1 *Family identity and offspring size measurements of the brown Arctic charr (Salvelinus alpinus) morph from lake Vatnshlíðarvatn sampled at two developmental stages hatching (H) and first feeding (FF).*

Family identity	H					FF			
	N	Mean SL (mm)	Range SL (mm)	CV (%)	DD	N	Mean SL (mm)	Range SL (mm)	CV (%)
16	8	15.5 ± 0.40	14.90-15.97	2.605	466	8	18.2 ± 0.30	17.65-18.61	1.669
18	5	13.5 ± 0.60	13.01-14.25	4.395	456	5	16.7 ± 0.50	16.06-17.26	2.969
24	4	13.4 ± 0.61	12.50-13.89	4.565	446	9	16.4 ± 0.56	15.51-17.38	3.422
25	8	15.9 ± 0.23	15.68-16.33	1.468	466	7	19.4 ± 0.25	19.08-19.78	1.265
28	5	17.2 ± 0.30	16.86-17.51	1.726	466	8	20.81 ± 0.49	20.20-21.78	2.348
30	5	14.3 ± 0.37	13.66-14.66	2.615	466	7	16.8 ± 1.02	15.11-18.19	6.076
31	7	14.3 ± 0.31	13.90-14.84	2.148	466	9	16.1 ± 0.72	15.56-17.80	4.451

Mean individual size (SL, standard length) and standard deviation (SD) of offspring used in this study were measured for all families. N, sample size; Range SL, range of standard length of offspring; CV, coefficient of variation; Degree days (DD) were sampled at 696 DD for all samples at FF.

All samples were stained simultaneously within each developmental stage and the same staining solution was used across both developmental stages to ensure as much consistency as possible. Once stained, individuals were placed in a petri-dish containing 2% and 3% of transparent methylcellulose (see Supplementary Info. S4.1) for H and FF respectively, to enable ease of maneuverability during photography and to ensure that the embryos lay flat. Individuals were photographed ventrally using a HD digital microscope camera (LEICA MC170 HD) mounted on a stereomicroscope (LEICA M165 C), with a scale set for each photograph. Individuals were photographed twice, each time removing and re-positioning the individual to account for any measurement error due to placement. The two sets of photos per individual were duplicated so each individual was represented by a total of four photos: two photos to account for placement error, and two photos to account for digitising error. Photos of disfigured individuals (N = 8) were removed and remaining photos randomised before digitisation. To capture phenotypic structure, we chose 17 fixed landmarks and 54 sliding semi-landmarks that were homologous between developmental stages, and placed on ventral surfaces of individuals in tpsDig2 v.2.31 (Rohlf, 2005; Collyer et al., 2015; see Fig. 4.1). The relative measurement error due to placement and digitising error was evaluated for each developmental stage using Procrustes ANOVA (Klingenberg et al., 2002) in MorphoJ (Klingenberg, 2011).

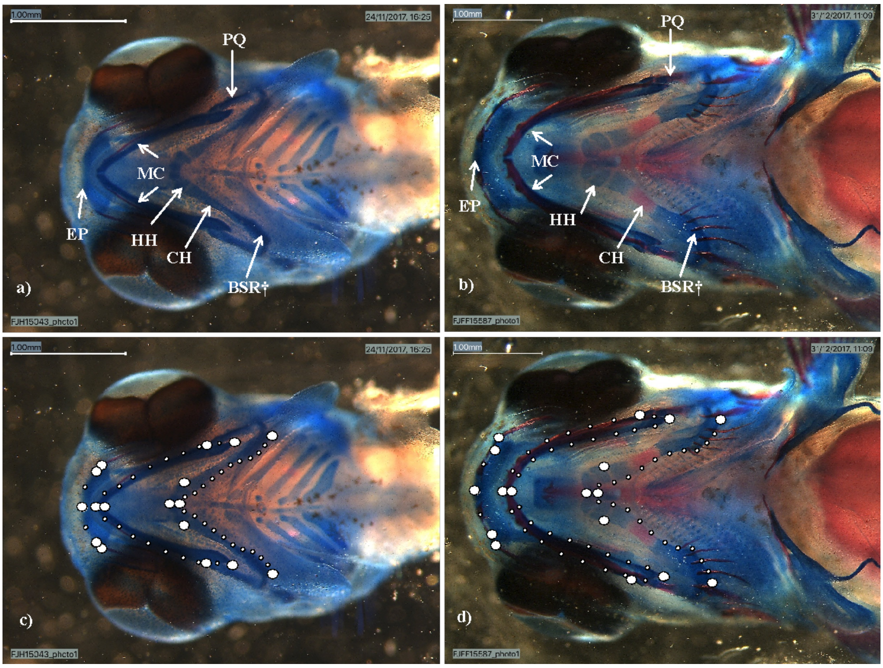

***Figure 4.1** Ventral craniofacial bones and cartilages at hatching (a) and first feeding (b) of Arctic charr (Salvelinus alpinus) embryos coming from the brown morph in lake Vatnshlíðarvatn Iceland. EP, ethmoid plate; MC, Meckel's cartilage; HH, hypohyal; CH, ceratohyal; BSR, branchiostegal rays; PQ, palatoquadrate. We refer to the combination of the HH, CH and BSR as the hyoid arch (HA). For timing and sequence of occurrence of these structures in Arctic charr see (Kapralova et al., 2015). The 71 homologous landmarks used to quantify craniofacial shape change at hatching (c) and first feeding (d) can be divided into fixed (larger dots) and semi-landmarks (smaller dots). † BSR were not considered, but are incorporated as part of the HA.*

4.3.3 Geometric morphometric analyses

All morphometric analyses were conducted in R v.3.3.2 (R Core Team, 2016) using the R package *geomorph* v.3.0.3 (Adams & Otárola-Castillo, 2013). Procrustes shape residuals were obtained from landmarks using a generalised Procrustes analysis (Rohlf & Slice, 1990), which optimally superimposes landmarks according to location, size and orientation. The resulting Procrustes residuals were then used for analysis of object symmetry (Klingenberg et al., 2002), using the function *bilat.symmetry* to average landmarks across the line of symmetry to remove variation due to side, as well as averaging our replicates. Using *procD.lm* we tested whether differences in shape were due to developmental stage, family-level variation, as well as the interaction of family and developmental stage. As *procD.lm* uses Type1 sum-of-squares, the position of the last variable was alternated in sequential regressions to obtain an unbiased estimate of the proportion of shape variance attributable to each variable. Since offspring size varied considerably between H and FF, we examined the effect of individual size on morphology, whilst controlling for any differences due to family, within developmental stage, using *procD.lm*.

We conducted a Principal component analysis (PCA) on the morphometric data. To visualise shape changes across families and developmental stages, we plotted up the first two axes from the PCA, showing morphological variation at the extremes of those distributions in relation to the average morphology using the function *plotTangentSpace*. We extracted scores of each individual on axes one and two and used them to determine the extent to which categorical variables (family + developmental stage) were related to shape variability across both developmental stages. A PCA was also conducted within developmental stage to test whether offspring size and/or family influenced shape variation. Given the small sample size of offspring within each family (N = 5-9), we are unable to test for allometric patterns within families (size-related changes of morphological traits; Klingenberg, 2016), but do examine how offspring size influences shape across families.

4.4 Results

Measurement errors were calculated for replicates using the Procrustes ANOVA. Variation due to placement accounted for 6% at H and 10% at FF, whilst variation due to digitising error was 3% and 6% at hatching and first feeding, respectively (Table S4.1). Once variation due to measurement errors, directional and fluctuating asymmetry were accounted for, actual variation between individuals accounted for 73% at H and 70% at FF (Table S4.1).

4.4.1 Craniofacial shape variation

Unbiased estimates of the shape variation determined by randomising the order of variables are shown in Table 4.2. The interaction between developmental stage and family identity was dropped from the model based on a lack of significance. Developmental stage did not affect craniofacial shape ($Z_{1,\,93} = 1.26$, $R^2 = 0.01$). The most variation in shape was explained by family identity alone, which explained 31% ($Z_{6,\,93} = 4.45$, $R^2 = 0.31$, $P = 0.001$) of the variation, but family did not affect shape when an interaction term with developmental stage was included. Visualisation of developmental stage and family effects on shape can be found in the PCA plot in Fig. 4.2. There were two clusters clearly visible in the PCA plot, with PC1 explaining 72.4% of the variation in shape, and PC2 explaining 15.7%. Although no clear separation of families nor developmental stage can be seen in these clusters, we found that family explained 34% of the variation found in PC1 ($Z_{6, 93}$ = 4.53, R^2 = 0.34, P = 0.001) where craniofacial shape goes from being very compressed with a blunted ethmoid plate but very wide Meckel's cartilage at the negative extreme, to a more elongated, larger and more narrow Meckel's cartilage at the positive extreme (Fig. 4.2). Additionally, family identity explained 35% of the variation in PC2 ($Z_{6, 93}$ = 4.66, R^2 = 0.35, P = 0.001), which was associated primarily with shape change in the ethmoid plate and hyoid arch, where the negative extreme shows a widening and elongation of the ethmoid plate whilst the hyoid arch becomes more narrow. In contrast, the positive extreme of PC2 shows a much wider hyoid arch and blunted ethmoid plate. To further understand to what extent family identity had on shape, each PC axis was plotted against family identity (Fig. 4.3). At the negative extreme of PC1, offspring of families 28, 30 and 31 have a more compressed shape with a wider Meckel's cartilage, whilst the positive extreme shows offspring from the remaining families to have a slightly wider and more elongated hyoid arch but narrower Meckel's cartilage (Fig. 4.3a). PC2 showed offspring from the same three families to also have a wider ethmoid plate and a compression of the Meckel's cartilage, with offspring from the other families showing a much wider hyoid arch (Fig. 4.3b).

Table 4.2 *Final Procrustes ANOVA model to determine the sequential significance of effects on craniofacial shape in Arctic charr. Significant effects are indicated in bold.*

	Df	SS	MS	Rsq	F	Z	P
Developmental stage	1	0.002	0.002	0.014	1.803	1.264	0.146
Family	**6**	**0.048**	**0.008**	**0.311**	**6.614**	**4.448**	**0.001**
Residuals	86	0.103					
Total	93	0.153					
Hatching							
Family	6	0.006	0.001	0.143	0.940	0.930	0.468
Size	1	0.000	0.000	0.006	0.245	0.205	0.833
Residuals	33	0.034					
Total	40	0.040					
First feeding							
Family	**6**	**0.020**	**0.003**	**0.225**	**2.193**	**1.783**	**0.029**
Size	1	0.001	0.001	0.009	0.544	0.380	0.513
Residuals	45	0.067					
Total	52	0.088					

Df, degrees of freedom; SS, sum of squares; MS, mean square; Rsq, r-square; F, F-value; Z, Z-value; and *P*, *P*-value of the significance of variables on influencing shape.

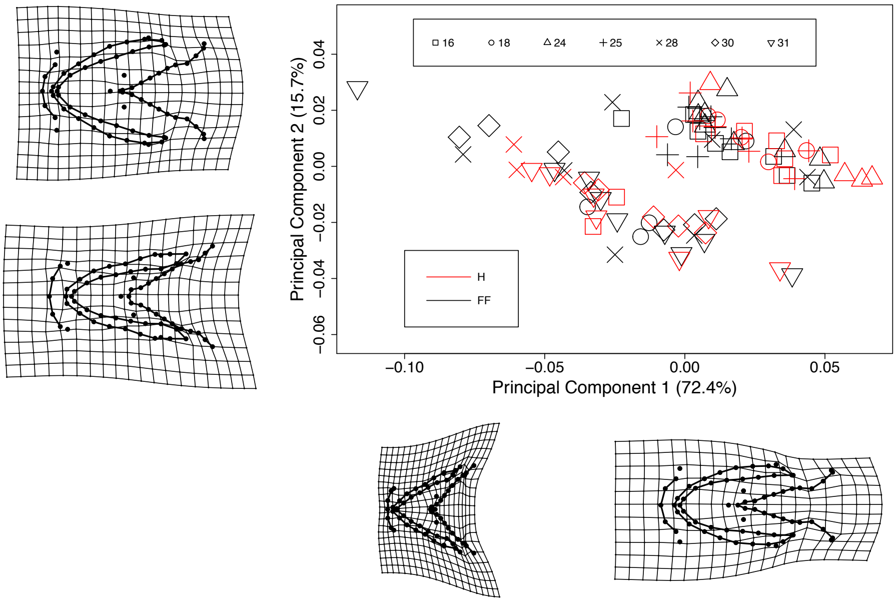

Figure 4.2 *Principal components analysis (PCA) plot showing craniofacial shape variation across both hatching (red) and first feeding (black) in the brown Arctic charr morph from lake Vatnshlíðarvatn. A total of 71 landmarks were used (see Fig. 4.1) and resulting deformation grids, with a 2x magnification, are presented at the extremes of both axes to facilitate the interpretation of shape change. Family identity is represented by different symbols.*

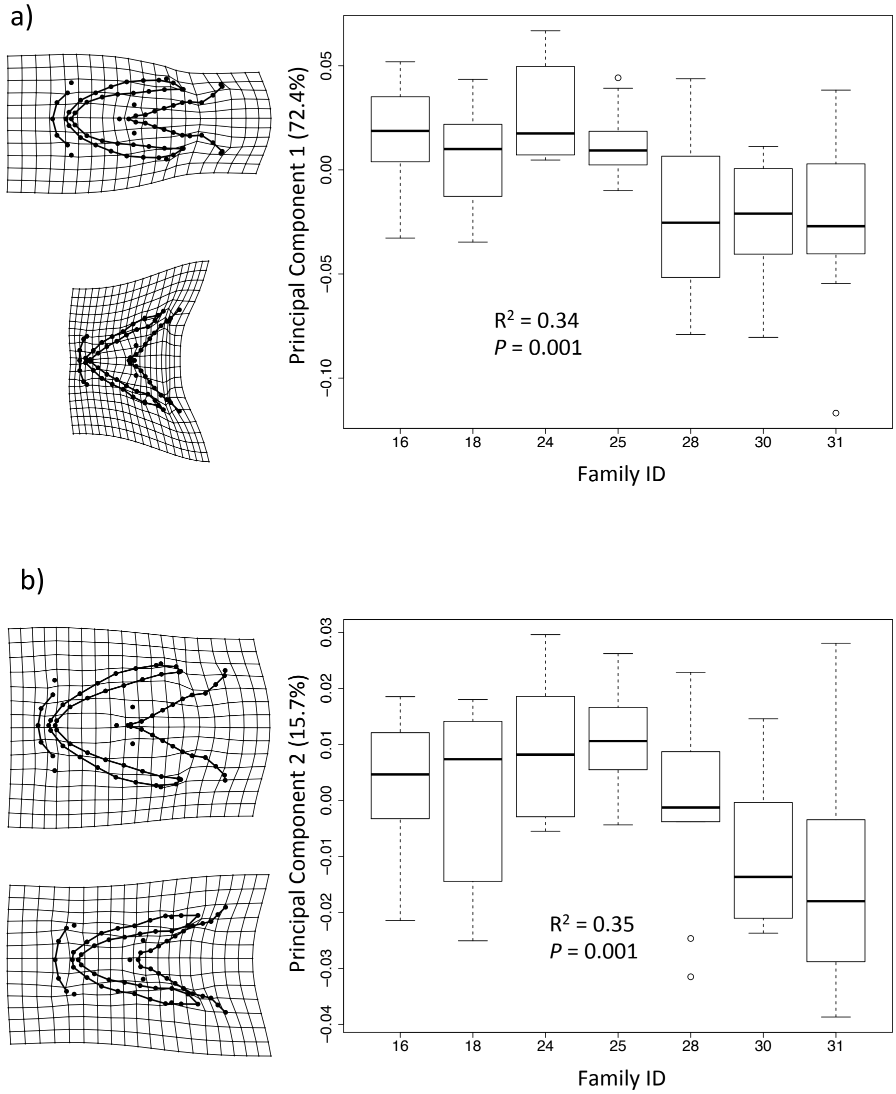

Figure 4.3 *Regression plots showing how head shape varies in the brown Arctic charr morph from lake Vatnshlíðarvatn with: a) PC1 and family identity (based on significant family effect across both developmental stages;* $Z_{6,\,93} = 4.53$, $R^2 = 0.34$, $P = 0.001$*) with families 28, 30 and 31 differing from all other families (*$P < 0.01$*) with the exception of families 18 and 28, and b) PC2 and family (also based on the significant family effect across both developmental stages;* $Z_{6,\,93} = 4.66$, $R^2 = 0.35$, $P = 0.001$*) with family 31 differing from all other families (*$P < 0.05$*) except 30, and family 30 differing from families 16, 24 and 25 (*$P < 0.05$*). Deformation grids are presented at the extremes of both axes to facilitate the interpretation of shape change with a x2 magnification.*

When examining data within each developmental stage, none of our variables explained any of the variation in PC1 or PC2 at H (Fig. 4.4a). At FF, an obvious separation existed between offspring from families 16 and 18 along both PC axis 1, explaining 72.3% of the variation, as well as PC2 (explaining 16% of shape variation; Fig. 4.4b). However, no association was found with either offspring size or family identity for PC1, but family did have a strong relationship with PC2 ($R^2 = 0.55$, $Z = 4.10$, $P = 0.001$) explaining 55% of the variation in shape. Indeed, offspring of two families (16 and 18) were clearly separated from the rest along PC2, having a wider head morphology and blunted ethmoid plate, in comparison to offspring of other families, which had a more narrow phenotype and elongated ethmoid plate (Fig. 4.4b).

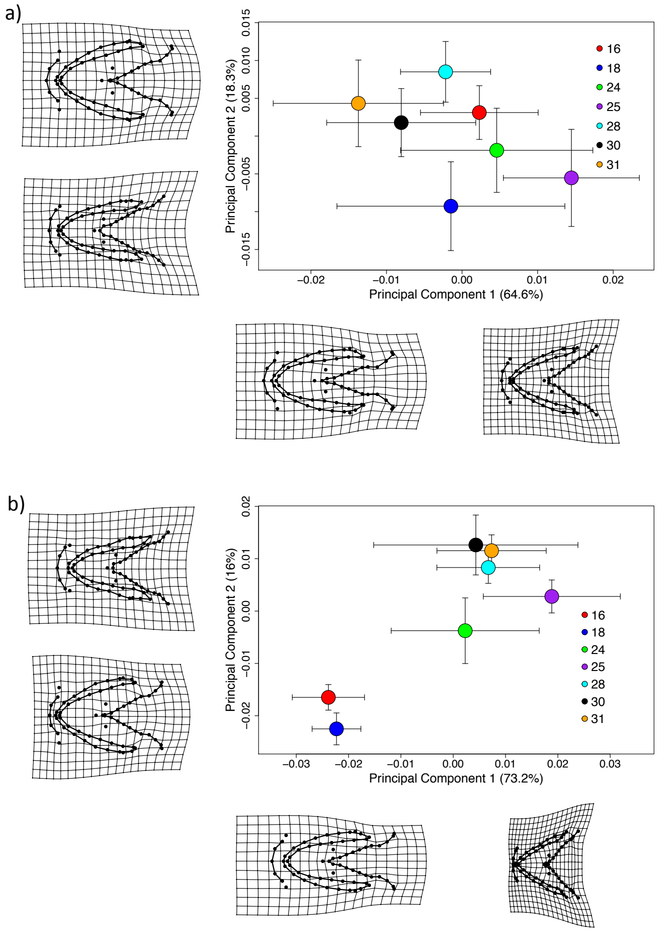

Figure 4.4 *Principal components analysis (PCA) plots showing shape variation of the brown Arctic charr morph from lake Vatnshlíðarvatn along each axis at two developmental stages: a) hatching, and b) first feeding. Coloured points and associated standard error bars correspond to mean shape of offspring per family. Deformation grids at the extremes of both axes represent the amount of shape change, with two-fold magnification.*

No differences in head shape were accounted for by individual size within each developmental stage (Fig. S4.1). However, the significant family effect at FF (Table 4.2) suggests that such analysis is not suitable at this developmental stage and should instead be conducted at a family level with higher numbers of offspring, which was not possible in our study.

4.5 Discussion

Our findings show considerable variation in the craniofacial morphology in progeny of the brown morph of Arctic charr in lake Vatnshlíðarvatn, early in development. Contrary to our hypothesis, there are no differences in shape associated with developmental stage. The results show that although there were no inter-individual differences in shape between offspring, there are strong differences in the shape of structures that are essential for feeding in offspring from different families at the first feeding stage. However, shape changes due to family were not noticeable at hatching. These strong family differences suggest that canalisation has not yet reduced the variation in phenotype in this weakly diverged, but more derived morph compared to the silver morph (Parsons et al., 2010, 2011). Family influences on offspring shape at first feeding demonstrates a role for maternal effects in mediating early-life stage variation in trophic structures, which have often been closely associated with population diversification (Skúlason et al., 1999; Adams et al., 2003; Reist et al., 2013). We primarily associate any variance due to family with maternal effects, since although paternal effects have been found to influence egg size in hatchery Arctic charr, there were no such paternal effects in salmonids from the wild (Pakkasmaa et al., 2001). These observable paternal effects in hatchery salmonids were attributed to the different environmental conditions experienced in the hatchery compared to the wild.

Trophic specialisation in Arctic charr has been found to influence morph divergence, even at contemporary timescales (Michaud, Power, & Kinnison, 2008). Indeed, gape width (which is a linear function of size in some fish species; Nilsson & Bronmark, 2000) can influence foraging ability in Arctic charr (Adams & Huntingford, 2002). Family identity was found to capture most of the variation of PC2 at first feeding (Fig. 4.4b) where the extremes vary from bluntening and widening, to elongating and narrowing of important structures involved in feeding and respiration, such as the hyoid arch (Fig. 4.1). A previous study on Arctic charr morphs from lake Þingvallavatn found offspring of the benthic morph to have a wider hyoid arch in comparison to offspring of the pelagic morph (Kapralova et al., 2015). In our study, offspring from most families have a relatively wide hyoid arch at first feeding (i.e. before plasticity has the chance to alter feeding morphology based on available external resources), similar to the benthic morph from lake Þingvallavatn (Kapralova et al., 2015). Having a wider hyoid arch might reflect different requirements for respiration, whereby benthic morphs have been found to have a larger respiratory surface area than the sympatric pelagic morph, which has been suggested to be linked with the lower dissolved oxygen levels in more benthic environments (Jenjan et al., 2018). However, such an explanation seems unlikely considering the oxygen rich ogliotrophic lakes found in Iceland. Additionally, the hyoid arch is also involved in support and extension of the jaw, and thus has the potential to influence prey choice in first feeding offspring. Only offspring from two families did not show this characteristic benthic craniofacial shape at first feeding. Strong family differences in offspring shape suggest that, within this morph, phenotypic variation is not canalised, providing further evidence that the morphs in lake Vatnshlíðarvatn are at the early stages of diversification, and likely closer to the ancestral phenotype (Parsons et al., 2011). This hypothesis, however, requires further study.

Despite much of the variation in shape at first feeding being attributed to family identity, we found no such relationship at hatching stage. Regardless of whether or not hatching is a stage (Kapralova et al., 2015) or whether it is a mechanism (Baroudy & Elliott, 1994; Valdimarsson et al., 2002), it seems that the release of embryos from the constraint of the egg permits the initiation of differences in developmental trajectories that, by the time first feeding occurs, has become large enough to be detected. By creating variability in developmental trajectories so that offspring differ in shape by first feeding, the success of offspring from different families in capturing prey of varying sizes increases, especially if food availability is unpredictable. In terms of temperature, Koops et al. (2003) found greater variability in egg sizes among females of brook trout (*Salvelinus fontinalis*) in less predictable environments. Such environmental heterogeneity, whether it is variability in food resources or variability in temperature, may therefore have the potential to drive variation in offspring traits that are then subjected to selection. Despite previous studies finding differences in the behaviour and feeding of small offspring, coming from smaller eggs, compared to large offspring from larger eggs a few weeks after first feeding (Benhaïm et al., 2003; Leblanc et al., 2011, 2016), we found no evidence to suggest that offspring size influenced craniofacial morphology in Arctic charr. However, it must be noted that this study was designed to examine variation in craniofacial shape based on offspring size alone, i.e. without the influence of plastic responses to diet choice. Yet it is this fine-scale variation in feeding structures that may be enhanced during the feedback between morphology, food, habitat choice and learned feeding specialisations that may not occur until later in development once the individual has been exposed to various prey/habitat types for longer (West-Eberhard, 2005).

Variability in structures related to feeding has the potential to influence what food resources are available to offspring with more narrow or wider trophic structures. Here we demonstrate how family effects can influence the shape of important respiratory and feeding structures of offspring, which has been linked to the evolutionary divergence of sympatric morphs (Skúlason et al., 1999; Adams et al., 2003; Reist et al., 2013). Trophic divergence may therefore occur, especially in environments where competition for limited food resources is high (Svanbäck et al., 2008). In these cases, variation in feeding structures during first feeding may facilitate divergence in resource use, resulting in increased intraspecific phenotypic variation, which may be further emphasised through plasticity, and possibly the evolution of resource polymorphisms (Hendry, 2009; Nosil et al., 2009; Schluter & McPhail, 1992; T. B. Smith & Skúlason, 1996). Anthropogenic pressures are causing major declines in biodiversity (Primack et al., 2018; Ripple et al., 2017), yet our understanding of the mechanisms forming and maintaining biodiversity is limited. By combining phenotypic and genetic studies along a diversification gradient, it should be possible to determine whether differential maternal investment (egg size) can ultimately act as a mechanism that may promote or precede the evolutionary origins of biodiversity.

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4.7 Appendix

Supplementary information S4.1 Acid-free double stain solution adapted from: Walker & Kimmel, 2006; Kapralova, 2014)

(*) = optional

Fixation

Embryos or larvae were fixed in 4% paraformaldehyde and stored in 100% Methanol at -20.

1. Transfer the embryos to 100% (or 96%) Ethanol (and rock at room temperature for 10 minutes.*)

Staining

Embryo (for larvae, skip to step 4):

2. Add 1 ml of acid-free Alcian blue, and 10ul of Alizarin red to each microfuge tube (mix before adding larvae), and rock at room temperature overnight

Bleaching

3. Remove the staining solution and add 1ml of water, mixby inversion and remove (x2*)
4. Add 1 ml of the bleaching solution and incubate for **20 minutes** at room temperature with the lids open. ## no rocking
5. (Add 1ml of water and mix by inversion*)

Clearing

6. Add 1 ml of a solution of 25% glycerol and rock at room temperature for 3 days for hatching (H) samples, or 4 days for first feeding (FF)
7. Replace with 1 ml of 50% glycerol and rock for 3 or 4 days for H and FF, respectively
8. Replace with 1 ml of 80% glycerol and rock for 3 or 4 days for H and FF, respectively

Photography

9. Prepare a solution of methylcellulose (2% for H, 3% for FF) and pour a specified amount into a petri-dish
10. Lay individual in methylcellulose so that the ventral surface points up to the camera and position accordingly

Storage

In a solution of 80% glycerol at 4°C.

Preparation of solutions

Alcian blue stock (0.4% Alcian blue in 70% ethanol)

For 100ml:

1. 0.04g of Alcian blue powder
2. Dissolve in 50% ethanol, incubated and stirred at 37°C
3. Add 95% ethanol and water to obtain final concentration

Alcian blue for sampling (part A)

1. Take 25ml of dH20 and dissolve 60 mM $MgCl_2$ (0,57126 g of $MgCl_2$); shake
2. Add 5ml of Alcian blue stock solution (0.4% in 70% ethanol)
3. Add 70ml 95% ethanol

Alizarin red (part B)

0,5% Alizarin red S powder dissovled in dH20.

Acid- free double stain solution contains 10µl of 0.5%Alizarin red (*part B*) and 1 ml of Alcian blue (*part A*) which are mixed just prior to staining. (Part A and B can be stored at room temperature (separately) for several months for up to a year.)

Bleaching solution

Mixed an equal volume of 3% H_2O_2 and 2% KOH to give final concentration 1,5% H_2O_2 and 1% KOH. NB: never use weak concentration of H_2O_2 as this will oxidise and will lower in concentration

Methylcellulose (2% for 100ml)

1. Heat up ~30mL 1xPBS in a beaker (~20s in microwave, the liquid should be very hot but not boiling)
2. Place ~70mL 1xPBS in a beaker, put the beaker on ice to cool
3. Weigh out 2g of methylcellulose powder, add to the hot 1xPBS
4. Stir until all powder is fully suspended, solution should look cloudy
5. Add ice cold 1xPBS (70mL) to the cloudy methylcellulose solution. Place entire beaker on ice and keep stirring for 2 hours - days (The solution will be very thick and gel like, so the stir bar may not spin in circles at this point, instead it will just keep shaking).
6. Make aliquots and store at 4 °C

***Table S4.1** Procrustes ANOVA of shape differences between replicated craniofacial measurements of Arctic charr embryos to calculate measurement error due to digitising error, placement of individuals as well as calculating variation due to fluctuating and directional asymmetry.*

Hatching stage					
Effect	**SS**	**MS**	**Df**	**F**	***P***
Individual	0.5948	0.0002	2960	4.89	**<.0001**
Side	0.0240	0.0003	74	7.9	**<.0001**
Ind*Side	0.1217	0.0000	2960	5.08	**<.0001**
Placement	0.0479	0.0000	5920	4.05	**<.0001**
Replicate	0.0239	0.0000	11988		
Total	0.8123		23902		
First feeding					
Individual	1.0598	0.0002	6512	7.11	**<.0001**
Side	0.0615	0.0008	74	36.31	**<.0001**
Ind*side	0.1490	0.0000	6512	2.07	**<.0001**
Placement	0.1441	0.0000	13024	3.18	**<.0001**
Replicate	0.0912	0.0000	26196		
Total	1.5056		52318		

Individual, variation attributable to actual differences between individuals after the effects of object symmetry and measurement error had been removed; Side, i.e. directional asymmetry, the average difference between the two sides of symmetry; Ind*Side, i.e. fluctuating asymmetry, the variability between left and right sides among individuals; Placement, variation due to placement of individuals in petri-dish; Replicate, variation due to digitising error. SS, sum of squares. MS, mean squares. Df, degrees of freedom. F, F-statistic.

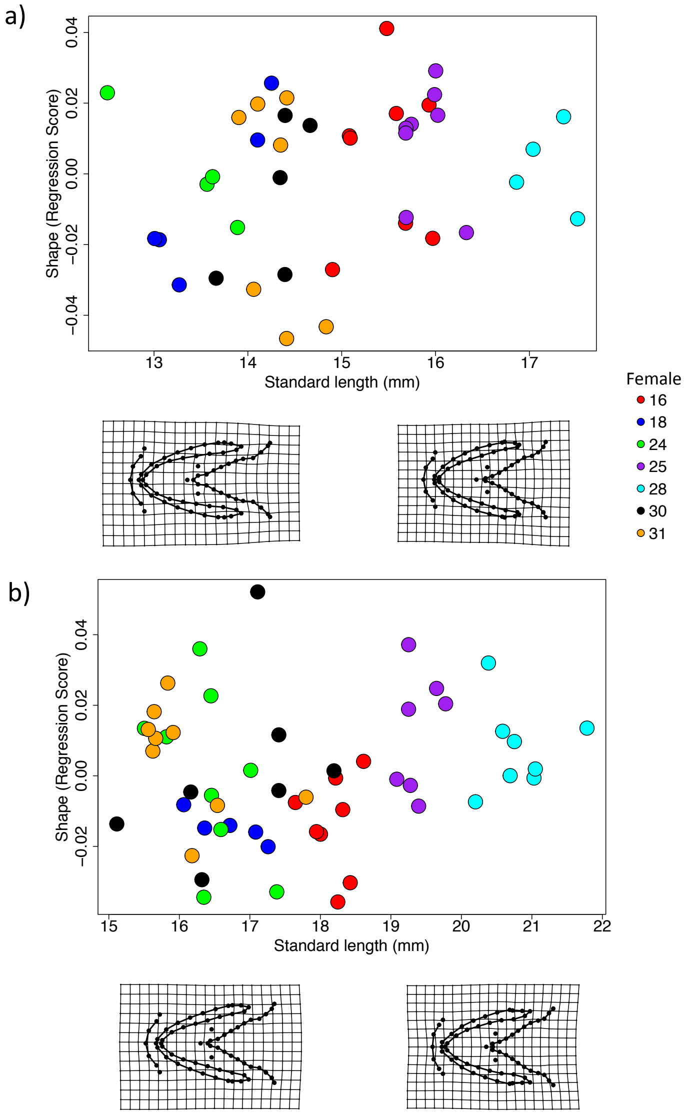

Figure S4.1 *The relationship between offspring body size (standard length, mm) and craniofacial morphology at a) hatching (H) and b) first feeding (FF). Deformation grids are 4x magnified and demonstrate the differences in shape change from the overall mean morphology at that particular developmental stage. Points are coloured according to family identity.*

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5 Paper IV

Egg size, gene expression and early life-stage phenotypic divergence in sympatric and allopatric Arctic charr (*Salvelinus alpinus*) morphs

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Authors' contribution: Conceived and designed the experiments: SVB CAL ZOJ BKK KR SS. Performed the experiments: SVB CAL AR. Analysed the data: SVB. Contributed reagents/materials/analysis tools: SVB CAL KR ZOJ BKK. Wrote the paper: SVB CAL KR BKK SS ZOJ.

5.1 Abstract

The mechanisms underlying the evolution of resource polymorphisms are poorly understood. Maternal effects are one such mechanism that has the potential to influence offspring developmental trajectories, but their influence on the phenotypic diversification in fishes have not been explored in depth. Here we aim to bridge this gap by determining the effects of egg size-correlated (relative) expression of genes related to growth and skeletogenesis on providing phenotypic variance along a gradient of sympatric divergence in Icelandic Arctic charr (*Salvelinus alpinus*). We ask whether gene expression (from post-fertilisation to first feeding) and craniofacial shape differ among morphs, and to what extent gene expression and craniofacial shape covary, as well as their connection to egg size. We particularly focus on traits and genes related to trophic morphology, and predict that 1) more derived morphs (relative to a putative ancestral anadromous population) have less variation (and thus less covariance) in shape, relative gene expression and their connection with egg size; 2) derived morphs are less similar to the ancestral population; and 3) weakly diverged sympatric morphs are more similar in shape and gene expression in comparison to the highly diverged sympatric morphs. Results found more derived morphs to have less variation in gene expression and shape, but are more *similar* to the ancestral population in relative gene expression, contrary to our prediction. Weakly diverged morphs were more different than the strongly diverged morphs in the number of genes differentially expressed, but not for craniofacial shape differences. In accordance with our prediction, egg size had the strongest effect on the weakly diverged and least derived Vatnshlíðarvatn silver morph. These results highlight the varying the influence maternal effects can have on offspring phenotype, the loss of which might be reflective of canalisation in more strongly diverged morphs.

5.2 Introduction

The extent of phenotypic and genetic divergence in natural populations can be arranged along a speciation continuum, ranging from small inter-individual differences, e.g. individual specialisation, to intra-specific polymorphisms and, finally, to complete reproductive isolation (Smith & Skúlason, 1996; Bolnick et al., 2003; Hendry, 2009; Nosil et al., 2009; Nosil, 2012). The specialisation of individuals in relation to different habitats or food resources can reduce resource competition and promote the coexistence of different morphs (i.e. resource polymorphism, Smith & Skúlason, 1996), thus facilitating the evolution of biodiversity through phenotypic and genetic differences between populations. However, the mechanisms bridging individual specialisation and speciation are not well understood. Understanding the sources of phenotypic variance and the processes driving phenotypic and genetic divergence is fundamentally important to bridge this knowledge gap.

Phenotypic variation was once considered to arise primarily from genetic change (Gilbert et al., 1996; Carroll, 2008). However, phenotypic changes are often plastic and can readily respond to environmental variation (Pigliucci, 2001). It is also becoming increasingly recognised that initial plastic phenotypic variation can facilitate evolutionary diversification (Price et al., 2003; West-Eberhard, 2003; Pigliucci, 2006; Pfennig et al., 2010; Moczek et al., 2011). The interplay between environmental heterogeneity and gene expression can influence phenotypic evolution by altering patterns of gene expression that are involved in the regulation of the phenotype (Gilbert, 2001), and allowing single genotypes to produce different phenotypes in different environmental conditions (an effect known as phenotypic plasticity, Pigliucci, 2001). Phenotypic plasticity (here used synonymously with developmental plasticity) is often (but not always; Pigliucci, 2001) fundamentally a developmental phenomenon, whereby different developmental pathways can produce continuous or discrete phenotypes (Frankino & Raff, 2004). Waddington's epigenetic landscape model demonstrates how an environmental or genetic stimulus (i.e. mutation) can alter developmental trajectories (i.e. the progression of certain developmental traits used to summarise the phenotype) so that the resulting adult phenotypes are best suited to their environment (Waddington, 1942, 1957). In some cases, selection acting at the developmental level reduces phenotypic variation (canalisation), leading to the persistence of the "acquired phenotype" regardless of changes in the environment or genotype (i.e. genetic assimilation; Waddington, 1942, 1953; Crispo, 2007; Parsons et al., 2011; Schneider & Meyer, 2017).

Teleost fishes provide some of the most spectacular examples of adaptive radiation and trophic polymorphism, where diversification of trophic structures plays a key role (Schluter, 1993; Skúlason et al., 1999; Hulsey et al., 2005; Seehausen, 2006; Turner et al., 2008; Takahashi & Koblmüller, 2011; Powder & Albertson, 2016). Such resource polymorphism is particularly common in freshwater fishes inhabiting post-glacial lakes in the northern hemisphere. The depauperate nature of northern lake ecosystems provides numerous habitats for individuals to specialise upon, thus promoting diversification through character release and resource polymorphism (Robinson & Wilson, 1994; Skúlason & Smith, 1995). Most cases of sympatric polymorphism in the northern hemisphere have been reported in salmonids (Fenderson, 1964; Ferguson & Mason, 1981; Lindsey, 1981; Skúlason et al., 1989a; Verspoor & Cole, 1989; Bernatchez et al., 1996; Woods et al., 2013), but similar examples have also been documented in other groups

(McPhail, 1984; Ehlinger & Wilson, 1988). Moreover, these resource polymorphic salmonids show extensive variation in the degree of phenotypic and genetic divergence in sympatry across lakes – which makes them exceptionally well-suited to study the mechanisms and processes underlying the origin and maintenance of diversity along a divergence continuum (Siwertsson et al., 2010; Seehausen & Wagner, 2014).

Along this divergence continuum in Arctic charr (*Salvelinus alpinus*), it was found that not only do offspring from more divergent morphs have lower levels of plasticity (Parsons et al., 2011), but also those morphs that are hypothesised to be more derived (relative to the putative ancestral anadromous population) exhibited a greater degree of canalisation (Parsons et al., 2010). The proximate mechanisms underlying the origins of such plasticity are, however, little understood (Ehrenreich & Pfennig, 2016). Given the strong effect on offspring phenotype and performance at early life stages, transgenerational plasticity (Mousseau & Fox, 1998; Agrawal et al., 1999) is one such mechanism that has the potential to influence developmental processes and thus intraspecific diversity. Maternal effects, the effects of the mothers' phenotype or environment on offspring phenotype and fitness (Wolf & Wade, 2009), are perhaps the best studied form of transgenerational effects and often arise from variation in egg size and content (Bernardo, 1996; Kaplan, 1998). In amphibians, for instance, differential egg size can influence plasticity, as well as divergence and maintenance of offspring traits involved in resource polymorphism (Kaplan, 1998; Kaplan & Phillips, 2006; Pfennig & Martin, 2009; Pfennig et al., 2010; review by Levis & Pfennig, 2018). In fishes, egg size often has strong effects on offspring phenotype, especially early in development, where larger eggs typically give rise to larger offspring (e.g. Chambers & Leggett, 1996; Heath & Blouw, 1998; Leblanc et al., 2016). Furthermore, egg size explained a large proportion of variation in early life-history traits in Chinook salmon (*Onchorynchus tshawytscha;* Thorn & Morbey, 2018), where offspring from smaller eggs had deeper bodies than offspring from larger eggs, which had a more fusiform shape (Cogliati et al., 2018). In brook trout (*Salvelinus fontinalis*), larger eggs had greater levels of plasticity in comparison to smaller conspecifics (Penney et al., 2018). Interestingly, although egg size is well recognised as a promoter of phenotypic variation in the early development of fishes, as to our knowledge, no study has yet explored the potential link between egg size-mediated phenotypic variation and evolutionary diversification of fishes. Although differences in the prioritisation of energy towards somatic growth or bone development is known to vary between offspring from different sized eggs (Eiríksson et al., 1999), very few studies have tested how egg size (i.e. maternally provided resources) mediated phenotypic differences translate into differential gene expression (Segers et al., 2012; Beck et al., 2018). In the cichlid *Simochromis pleurospilus*, egg size influenced expression levels of a growth hormone receptor (*GHR*), which was accompanied by compensatory growth in offspring originating from small eggs with higher *GHR* after hatching (Segers et al., 2012). In Arctic charr, egg size correlated with relative expression of a few genes involved in the promotion of skeletogenesis (*Mmp9*) and growth (*Sgk1* and *Star*; Beck et al., 2018, *Paper II*). These same genes have been shown to be associated with craniofacial divergence between Arctic charr morphs (Ahi et al., 2014). Maternal effects (i.e. egg size) and offspring gene expression have the potential to jointly influence plasticity, but how they collectively influence the phenotype and potentially resource polymorphisms, has not been studied.

Arctic charr is a well-suited species for studying determinants of variation along a divergence continuum. This species occurs in numerous lakes where allopatric and sympatric resource morphs vary in their degree of phenotypic and genetic divergence, having anything from 1-4 morphs residing in sympatry (Magnusson & Ferguson, 1987; Skúlason et al., 1989a; Gíslason et al., 1999; Jónsson & Skúlason, 2000; Noakes, 2008; Klemetsen, 2010; Skoglund et al., 2015), where the more benthic and/or more specialised morphs are considered to be more derived (Parsons et al., 2011). Arctic charr also vary substantially in both mean and variability of egg size within and among females, as well as among morphs (*Chapter I*). Here, we test to what extent egg size may influence phenotypic variance in both gene expression and craniofacial shape in five morphs of Arctic charr that vary in their degree of sympatric divergence. We combine data on variation in egg size, offspring phenotype (tropic morphology) and relative expression of genes associated with craniofacial divergence and growth (Ahi et al., 2014; Beck et al., 2018). Using geometric morphometrics and qPCR, we compare a weakly and a strongly divergent sympatric pair to a putative ancestral population (i.e. anadromous Arctic charr), and ask whether: 1) offspring craniofacial shape and relative gene expression differ among morphs along a divergence continuum, 2) there is any covariance between changes in relative gene expression and also changes in craniofacial shape of offspring; and 3) this variation in relative gene expression, craniofacial shape, and their covariance is related to egg size. We focus on phenotypic traits and genes related to trophic morphology and predict that more derived morphs (i.e. þingvallavatn large benthic and Vatnshlíðarvatn brown) – relative to the ancestral population – show more canalised shape and gene expression (Parsons et al., 2011). We also expect the weakly diverged morphs from lake Vatnshlíðarvatn to be more similar to each other in both gene expression and shape in comparison to the highly diverged sympatric morphs from lake þingvallavatn (Gíslason et al., 1999; Kapralova et al., 2011). Previously, using a single morph that displayed the most variation in egg size (Vatnshlíðarvatn brown), we found no evidence for egg size to influence craniofacial shape (*Paper III*), but did find some evidence (albeit weak) to suggest that egg size can be correlated with relative gene expression (Beck et al., 2018, and *Paper II*). We therefore expect to find no differences in craniofacial shape due to egg size, but do expect to find the relative expression of genes related to skeletogenesis and growth to be correlated with egg size. Finally, we predict that any covariance between gene expression and craniofacial shape will be found in those weakly diverged morphs (from lake Vatnshlíðarvatn) due to their less canalised developmental trajectories.

5.3 Materials and methods

5.3.1 Study system, rearing and sampling

Four groups of individuals with similar morphologies (i.e. behaviour and trophic apparatus) – here referred to as morphs – and one anadromous population of Arctic charr were used in this study (Fig. 5.1; see *Paper I* for a more comprehensive description of all the morphs). 1) Fljótaá, an anadromous population reflecting the putative ancestral population. 2) Two morphs (silver and brown) within lake Vatnshlíðarvatn, a physically simple, small and shallow lake (Jónsson and Skúlason, 2000) with Arctic charr being the only fish species present. The brown morph (VB) has a more specialised diet in comparison to the silver morph (VS), which retains its ancestral migratory behaviour of spawning in streams (Jónsson and Skúlason, 2000; Parsons et al., 2011). 3) Þingvallavatn, Iceland's largest lake, where Arctic charr has evolved in to four morphs (large benthic, small benthic, planktivorous and piscivorous). For our study, we use the large benthic (TLB) and the planktivorous (TP) morphs, which are known to differ in shape from very early in development (Skúlason et al., 1989a; Kapralova et al., 2015). All morphs were caught in the years 2014-2016 (FJ = 2016, VB = 2014, VS = 2015, TLB = 2014 for gene expression and 2015 for shape [this sampling design results in TLB being dropped from the analyses when combining gene expression and craniofacial shape data], and TP = 2016), with those that spawned within lakes collected using gill nets, and river (FJ) / stream (VS) spawners collected using electrofishing.

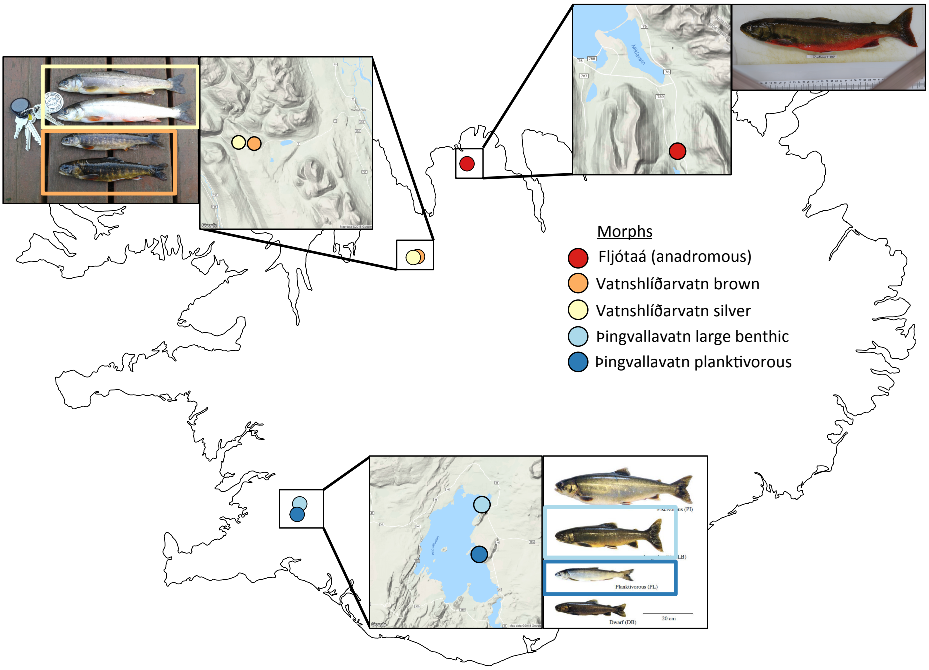


Figure 5.1 *Map of all locations of Arctic charr (Salvelinus alpinus) sampled in this study across Iceland (modified from Beck et al., Paper III). In cases where multiple morphs co-exist, those used in this study are highlighted. Vatnshlíðarvatn silver and Fljótaá (anadromous) both migrate upstream/river (respectively) to spawn. Map of spawning grounds are at the same scale.*

Single pair matings were conducted in the field (see Table 5.1 for sample sizes), where fertilised eggs were allowed to water-harden before transport to Hólar University College's aquaculture facilities in Verið, Sauðárkrókur. Embryos were reared in cages (6.5cm x 6.5cm x 5cm) with two 2cm x 2 cm holes at the front and rear. The holes and the bottom of the cage were covered with mesh to enable good oxygenation. Cages were placed in a shelf incubator system (MariSource 8-tray Vertical Incubator) with a constant flow of 95% recycled water. For some families, a single cage was used (one layer of eggs), but when eggs were numerous they were split into different cages. Embryos were reared in darkness to mimic natural conditions, and constant temperature (4.2ºC ± 0.48 SD), measured using HOBO loggers (to the nearest 0.001°C) four times daily. Dead individuals (opaque eggs) were manually removed every two days to prevent fungal growth. An accumulative temperature estimate (degree days, DD; Pruess, 1983) was used to determine the approximate time by which certain developmental stages would occur (see below). Individuals were digitally photographed (Canon EOS 650D) alongside a length scale before fixation or placement in RNA later, and standard length (SL) or egg diameter (Leblanc et al., 2016) measured using the program Fiji (Schindelin et al., 2012).

5.3.2 RNA extraction and cDNA synthesis

For gene expression studies, animals were collected and sacrified (see *Paper II* for details), then measured using qPCR at: 1) before zygotic gene expression (i.e. before the mid-blastula transition, MBT; Kane & Kimmel, 1993), which is known to occur at 42 DD post-fertilisation (PF) in Atlantic salmon (*Salmo salar*; Nagasawa et al., 2013); 2) eye stage (E), when 50% of embryos in a given cage had eye lenses visible; 3) when 50% of embryos within a cage had hatched (H); and 4) when 50% of embryos had started first feeding (FF). At the FF stage, individuals were decapitated behind the pectoral fin and RNA extracted from the head, as much of the phenotypic variation in Arctic charr is associated with trophic morphology, as well as large body size being reflected by large head size (Kapralova et al., 2015). Immediately after collection, samples from all four stages were stored in an RNA stabilising buffer (De Wit et al., 2012) and pre-incubated at room temperature before storage (-20ºC).

Tissues were homogenised using Bead Beater (Biospec) and total RNA extracted using TRI reagent (Sigma-Aldrich, St Louis, MO, USA). RNA extraction and cDNA synthesis were conducted according to Ahi et al. (2013). The RNA was precipitated using isopropanol, washed with ethanol and air-dried. The RNA pellet was resuspended in RNase-free water and the RNA yields quantified using NanoDrop ND-1000 UV/VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The samples were treated with DNase I (New England, Biolabs, Ipswich, MA, USA) to remove any contaminating DNA. A subset of extracted RNAs were electrophoresed on agarose gels to confirm RNA quality. Single stranded cDNA was synthesised from 1 µg of total RNA, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. cDNA was consequently diluted in nuclease-free water in preparation for qPCR.

Fourteen genes were chosen for analyses of early life-stage gene expression based on their previously demonstrated involvement in growth or skeletal development (see Beck et al., 2018 [*Paper II*] for full descriptions of genes). We used two validated early developmental Arctic charr reference genes (*Actb* and *Ef1a*) for qPCR data normalisation based on Ahi et al. (2013). The following target growth-promoting genes were selected and described in full in our previous study (Beck et al., 2018), which includes: *Star,* Steroidogenic Acute Regulatory Protein, chosen based on its involvement in craniofacial divergence in Arctic charr (Ahi et al., 2014); *Igf1* and *Igf2,* insulin-like growth factors; *Nr3c1* – here called *Gr* – glucocorticoid receptor; *Mtor,* mechanistic target of Rapamycin; *Sgk1,* serum glucocorticoid kinase; *Rictor*, rapamycin-insensitive companion of mammalian target of rapamycin; and *Ghr1*, growth hormone receptor. Six target genes involved in promoting skeletogenesis (chosen based on their divergent expression in the developing heads of Arctic charr morphs: Ahi et al., 2014): *Timp2*, tissue inhibitor of metalloproteinases 2; *Ets2,* V-Ets Erythroblastosis Virus E26 Oncogene Homolog 2 (Avian); *Sparc,* Secreted

Protein, Acidic, Cysteine-Rich (Osteonectin); *Ctsk,* Cathepsin K; *Mmp2*, Matrix Metallopeptidase 2; and *Mmp9,* Matrix Metallopeptidase 9 (see Beck et al., 2018). These extra-cellular matrix (ECM) re-modelling genes are essential for skeletogenesis (with roles in trophic skeletogenesis) and make up a highly conserved co-expression network across vertebrates, regulated by transcription factors such as *Ets2* (Ahi et al., 2014).

5.3.3 qPCR and normalisation

Primers (Beck et al., 2018) were designed using an assembled Arctic charr transcriptome (Guðbrandsson et al., 2018) and exon boundaries mapped to *Salmo salar* orthologs from the salmonid species database (Di Genova et al., 2011). Primers spanned at least one exon boundary to avoid amplifying DNA and were selected based upon their short amplicon size (<250bp). Primer efficiencies were calculated (Beck et al., 2018) and both RT-qPCR and differential mRNA gene expression calculations for each target gene performed according to Ahi et al. (2014), using two validated reference genes, *Actb* and *Ef1a*, which were normalised using the first individual as a calibrator sample for each population and each developmental stage. Relative expression quantities (RQ) were calculated according to Ahi et al. (2014).

Table 5.1 *Sample sizes (N) and female (♀) trait means for the anadromous population and four morphs of Arctic charr. Depicted are numbers of offspring used for relative gene expression (GE) at four developmental stages (post-fertilisation (PF), eye stage (E), hatching (H) and first feeding (FF)), and for staining at two developmental stages (H and FF). The morphs include 1) FJ, Fljótaá; 2) VB, Vatnshlíðarvatn brown; 3) VS, Vatnshlíðarvatn silver; 4) TLB, Þingvallavatn large benthic; and 5) TP, Þingvallavatn planktivorous.*

Morph	Family ID	♀ FL (cm)	♀ age	♀ egg size (mm)	PF		E		H				FF			
					Off. N GE	DD	Off. N GE	DD	Off. N GE	Off. N Stain	DD	Off. SL (mm)	Off. N GE	Off. N Stain	DD	Off. SL (mm)
VB	16	17.6	5	4.6 ± 0.28	5	27	8	234	7	8	464	14.7 ± 1.21	5	8	657	19.0 ± 1.29
VB	18	14.5	5	3.8 ± 0.29	6	27	4	234	6	5	456	14.3 ± 1.40	7	5	654	18.1 ± 1.61
VB	24	15.2	6	3.8 ± 0.24	6	27	3	234	5	4	462	13.2 ± 0.37	4	9	632	17.6 ± 1.80
VB	25	18.6	6	4.8 ± 0.37	5	27	3	234	6	8	460	15.1 ± 0.08	5	7	659	17.7 ± 1.45
VB	28	20.6	8	5.4 ± 0.46	5	27	6	234	6	4	463	15.8 ± 1.09	8	8	665	18.4 ± 1.84
VB	30	14.7	4	4.3 ± 0.54	6	27	5	234	6	5	464	14.5 ± 1.42	6	7	666	17.1 ± 0.99
VB	31	14.2	4	4.0 ± 0.29	6	27	6	234	4	7	458	15.1 ± 1.14	6	9	670	16.5 ± 1.56
VS	180	25	6	4.4 ± 0.18	6	5	6	220	6	15	391	13.2 ± 0.50	6	10	655	18.6 ± 0.82
VS	182	23	5	4.2 ± 0.18	6	5	6	220	5	7	391	12.9 ± 0.71	5	13	655	17.3 ± 0.99
VS	184	24.2	6	4.6 ± 0.17	6	5	6	220	6	7	403	14.3 ± 0.38	6	11	639	19.1 ± 0.76
VS	191	24	6	4.8 ± 0.17	6	5	6	223	6	5	407	14.5 ± 0.23	6	10	631	19.5 ± 0.74
VS	194	24	5	4.8 ± 0.28	6	5	6	220	6	6	416	15.2 ± 0.66	6	11	626	19.6 ± 0.89
FJ	308	35.6	3	4.5 ± 0.40	4	10	6	199	6	10	443	15.7 ± 0.89	6	20	649	20.6 ± 0.78
FJ	311	46	4	4.8 ± 0.12	5	10	6	199	6	8	441	16.5 ± 0.36	6	20	649	20.9 ± 0.53
FJ	313	37.8	3	4.3 ± 0.11	6	10	6	204	6	12	435	15.4 ± 0.44	6	9	649	19.2 ± 0.90
FJ	315	32.5	4	4.4 ± 0.17	2	10	5	204	6	9	441	15.6 ± 0.47	6	10	649	20.9 ± 0.73
FJ	339	32.4	5	4.5 ± 0.21	4	10	6	198	6	10	433	15.8 ± 0.74	5	21	617	19.5 ± 0.69
FJ	342	34.7	3	4.3 ± 0.26	4	10	5	198	5	6	431	15.4 ± 0.83	5	10	612	19.8 ± 0.74
TLB	2	30	8	5.1 ± 0.20	4	14	2	212	5		443	16.3 ± 0.43	6		680	20.4 ± 0.71
TLB	3	35.2	6	4.8 ± 0.20	6	14	8	212	5		450	15.9 ± 1.29	6		677	20.9 ± 1.04
TLB	6	36.1	10	5.0 ± 0.19	5	14	7	212	3		421	16.2 ± 0.70	7		672	20.5 ± 0.58
TLB	10	28.5	11	5.0 ± 0.14	5	14	4	212	4		424	16.3 ± 0.90	5		665	20.7 ± 0.62

TLB	11	37	11	5.1 ± 0.18	7	14	8	212	4		436	16.7 ± 0.34	4		656	21.4 ± 0.68
TLB	116	40.3	8	5.2 ± 0.25		6		220		12	445	19.5 ± 0.76		5	643	14.6 ± 1.52
TLB	119	39.1	7	4.9 ± 0.14		6		220		16	425	15.9 ± 0.97		5	643	18.7 ± 0.77
TLB	128	40	8	4.6 ± 0.31		6		220		10	432	15.2 ± 0.62		6	660	19.4 ± 2.17
TP	320	19.9	5	4.7 ± 0.21	5	5	6	200	6	9	457	16.4 ± 0.74	6	11	630	19.6 ± 1.03
TP	321	19.4	6	5.0 ± 0.14	5	5	5	200	6	8	450	17.3 ± 0.48	6	9	630	21.4 ± 0.90
TP	322	22.3	5	5.4 ± 0.11	5	5	5	200	6	6	450	18.3 ± 0.40	6	10	630	23.1 ± 0.68
TP	324	23.7	7	5.2 ± 0.15	5	5	5	200	6	8	453	18.0 ± 0.64	6	9	630	22.2 ± 1.21
TP	325	19.7	6	4.9 ± 0.23	6	5	6	200	6	9	453	16.8 ± 0.58	6	10	630	20.9 ± 0.65
TP	329	20.2	6	4.9 ± 0.19	6	5	6	200	6	8	453	17.2 ± 0.67	6	9	630	21.1 ± 0.66

Female fork length (FL), age (in years), mean egg size and associated standard deviations (±) are shown, as well as mean degree days (DD) and standard length (SL) of offspring (Off.) at each developmental stage. Blanks for TLB are due to TLB not having both gene expression and staining data for the same families due to sampling error.

5.3.4 Staining and digistisation

To characterise shape and size between morphs, and to test whether egg and/or individual size affected craniofacial morphology, a total of 181 offspring from H stage, and 219 offspring from FF stage were selected from a total of 20 families (see Table 5.1). Individuals were fixed in 4% paraformaldehyde (PFA) and stained red (Alizarin) for bone and blue (Alcian) for cartilage, using a modified protocol from (Walker & Kimmel, 2007; Kapralova, 2014; see *Paper III*).

Based upon a previous study by Kapralova et al. (2015), as well as results from Beck et al. (*Paper III*), we selected craniofacial elements that were clearly stained and visible at both H and FF stages (see Fig. 5.2). For a comprehensive overview of all structures visible in developing embryos of Arctic charr, as well as the timing of appearances of craniofacial cartilages/bone in developing arctic charr, see Kapralova et al., (2015). Similar to *Paper III*, all individuals were photographed according to Beck *et al.* (*Paper III*), where two sets of photos of the same view per individual were duplicated so each individual was represented by a total of four photos: two photos to account for placement error, and two photos to account for digitizing error. To facilitate detection of phenotypic variation, we chose 17 fixed landmarks and 54 sliding semi-landmarks that were homologous between developmental stages, and located on ventral surfaces of individuals in tpsDig2 v.2.31 (Rohlf, 2005; Collyer et al., 2015), see Fig. 5.2. The relative amount of measurement error due to placement and digitising was measured for each developmental stage using Procrustes ANOVA (Klingenberg et al., 2002) in MorphoJ (Klingenberg, 2011). To minimise variation due to measurement errors and asymmetry, we used *bilat.symmetry* in the *geomorph* package v.3.0.3 (Adams & Otárola-Castillo, 2013) in R (R Core Team, 2016), which averages landmarks across replicates as well as between the left and right sides of a given individual (Klingenberg et al., 2002).

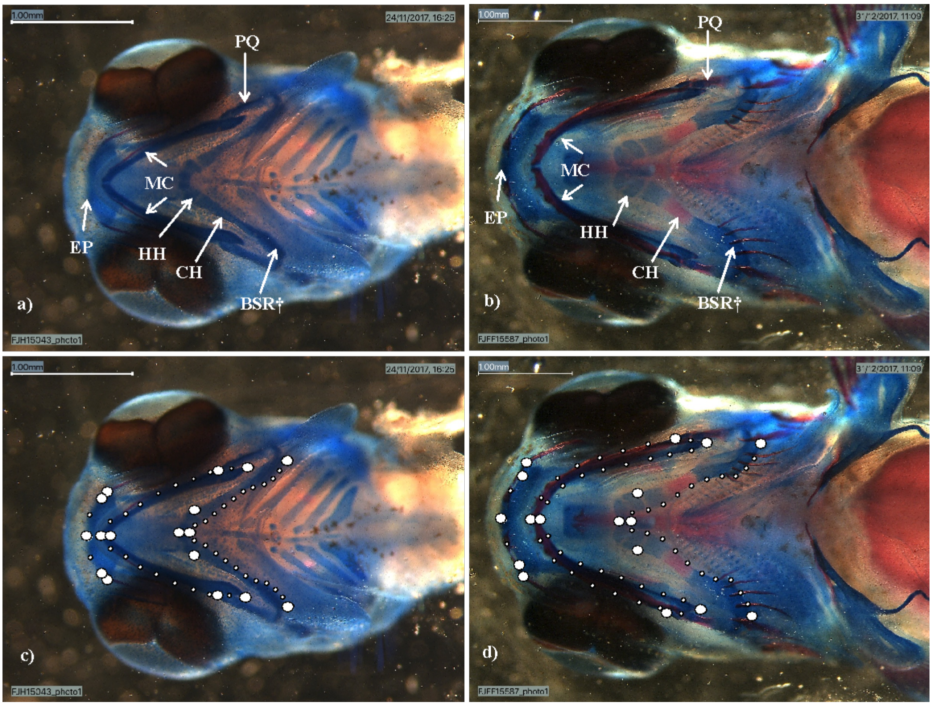


Figure 5.2 *Craniofacial elements of Arctic charr (Salvelinus alpinus) at hatching (a) and first feeding (b), taken from Beck et al., Paper III. EP, ethmoid plate; MC, Meckel's cartilage; HH, hypohyal; CH, ceratohyal; BSR, branchiostegal rays; PQ, palatoquadrate. We refer to the combination of the HH, CH and BSR as the hyoid arch (HA). For timing and sequence of occurrence of these structures, see Kapralova et al. (2015). The 71 homologous landmarks used to quantify craniofacial shape change at hatching (c) and first feeding (d) can be divided into fixed (denoted by larger point) and semi-landmarks (smaller points). † BSR were not measured, but the structures in which they are attached were.*

5.3.5 Statistical analyses

The analyses were done in several steps to test our predictions. First, we tested how offspring gene expression varied across morphs (unless otherwise specified, for the Analyses and Results we include the ancestral anadromous population under the term 'morph', for brevity) and developmental stages, using individual-level data, as well as including data from VB (Beck et al., 2018, *Paper II*). When significant morph effects were found, we subsequently conducted analyses within morphs to test how developmental stage, and individual size affected gene expression. We then conducted shape analyses on family means (due to small sample sizes within families) across morphs followed by pairwise comparisons if the morph effect was significant. Analyses comparing how head shape varied with gene expression were also conducted on family means (N = 5 to 7 / morph), as gene expression and shape variation were not assessed on the same individuals (note that TLB was dropped from this part of the analyses as the same families were not collected for both shape and gene expression).

All statistical analyses were performed in R v.3.3.2 (R Core Team, 2016), with morphometric analyses conducted using the R package *geomorph* v.3.0.3 (Adams et al., 2018). For the gene expression data, all analyses were performed on $\log_2$ transformed relative quantification (RQ) values. Normality was investigated by examining the residuals of each model using quantile-quantile (QQ) plots and histograms. *Post-hoc* pairwise comparisons of adjusted means and slopes (where significant interactions exist) were conducted using the R packages *lsmeans* (Lenth, 2016) and *emmeans* (Lenth, 2017), respectively.

5.3.6 Gene expression among and within morphs and developmental stages

To test for morph differences in gene expression across developmental stages, we used morph (5 levels), developmental stage (4 levels), gene identity (14 levels) as well as their two and three way interactions as fixed effects. Family and cage (nested within morph) were used as random effects in all models of the gene expression analyses to account for non-independence. Due to a significant three-way interaction between morph, developmental stage and gene identity (see Table S5.1), subsequent analyses were conducted within each of the four developmental stages (PF, E, H and FF) to test for variation among morphs in gene expression. These analyses were conducted using linear mixed effects models (LME) fitting the *lmer* function from the *lme4* package (Bates et al., 2015). In these models, gene identity and morph as well as their interaction were used as a fixed effect, and family and cage (nested within morph) as random effects. To further disentangle any significant differences in morph differences in gene expression, LME models were finally conducted within each gene and developmental stage, with morph as a fixed effect.

To understand how gene expression varies within morphs, we included an interaction between developmental stage and gene identity. Since within morph variation in gene expression of the VB morph are presented in Beck et al. (2018), they are not described much in the results section, however they are shown in the figures. We further assess any significant interactions by examining expression levels per gene across developmental stages. (Note, however, that any differences in gene expression at FF compared to other developmental stages may in part be linked to our sampling procedures, as RNA was extracted from the whole embryo at PF, E and H, but only from the head at FF stage; Beck et al., 2018). All morphs had family (synonymous with ‘female’ used in Beck et al. [2018]) and cage included as a random effect, except for TP, where cage was not included as a random effect as eggs from each family were all placed in a single cage.

5.3.7 Size-correlated gene expression within morphs

To test for dependency of gene expression with individual size, LME models were fitted within each developmental stage and morph with size as a covariate. These models included gene identity (14 levels) as a fixed factor and individual size at a given level, as well as its interaction with gene identity. As above, family identity and cage were included as random effects except for the TP morph at all developmental stages, and for VS at PF, where only family was used as a random effect as all offspring were placed in a single cage. The *anova* function was used to determine whether the best fitting model (within a given developmental stage) was linear or polynomial (second or third order) using Akaike

Information Criterion, AIC (e.g., Zuur, 2009). Subsequent analyses were then conducted using either the linear or non-linear model. If the size × gene identity (or size^x × gene identity) interaction was significant, the slopes of gene expression and individual size were compared across all genes to determine how expression of genes was correlated with size using the *test* function, which tests which slopes differed significantly from zero. The *pairs* function from the estimated marginal means (*emmeans*) package (Lenth, 2017) was then used to test for pairwise comparisons between slopes.

5.3.8 Geometric morphometric analyses

All morphometric analyses were conducted using the R package *geomorph* v.3.0.3 (Adams & Otárola-Castillo, 2013) on family means due to small sample sizes within families (minimum number of individuals per family = 3). Procrustes residuals were obtained from landmarks using a generalised Procrustes analysis (Rohlf & Slice, 1990), which optimally superimposes landmarks according to location, size and orientation. The resulting Procrustes residuals were then used for analysis of object symmetry (Klingenberg et al., 2002). We performed a Procrustes ANOVA examining the interaction between dev.stage × morph to determine whether craniofacial shape differed between morphs and developmental stages. We subsequently conduct all shape analyses within each developmental stage.

5.3.9 Egg size and craniofacial shape differences between morphs

To visualise shape differences across morphs, we calculated the overall mean shape as well as the mean shape within each morph. Subsequently, deformation grids of each morph at each developmental stage were plotted in relation to overall mean shape. Based on the significant correlation between egg size and offspring size (t = 2.89, P = 0.006), we use egg size as the measure of allometry. Using *advanced.procD.lm* on Procrustes residuals from *bilat.symmetry,* we tested whether differences in shape were related to morph and developmental stage, as well as whether mean egg size of offspring per family influenced shape (i.e. testing craniofacial shape differences both before and after accounting for allometry). Furthermore, to determine whether mean egg size size per family influenced shape differently in different morphs and developmental stages, we examined the interaction term between these three variables (mean egg size, morph, and developmental stage). Based on significant differences in craniofacial shape between developmental stages, further analyses were conducted within developmental stages, where we test for differences in craniofacial shape across and within morphs both before and after accounting for differences due to egg size (i.e. allometry). Any significant effects of egg size on craniofacial shape were plotted using *procD.allometry.*

5.3.10 Covariance between gene expression and craniofacial shape

Partial least squares (PLS; Rohlf & Corti, 2000) was used to assess whether craniofacial shape covaries between both the relative expression of genes related to skeletogenesis, as well as growth within each developmental stage, and across and within morphs (excluding TLB due to sampling error). Two-block PLS examined patterns of covariation between craniofacial shape (Procrustes residuals) and standardised relative expression of a) genes involved in skeletogenesis, and b) genes involved in growth. A singular value decomposition is obtained from the two covariance matrices (craniofacial shape and relative gene expression), whereby the resulting PLS axes are uncorrelated, where the first axis explains maximal covariation between both blocks (gene expression and shape; Rohlf & Corti, 2000; Young et al., 2016). The amount of covariation between the two blocks was measured using a multivariate correlation coefficient (r_{PLS}: Adams & Collyer, 2016), with associated P-values based on 10,000 permutations under the null hypothesis of independence between both blocks of variables. The resulting PLS components have high covariance with the response, and those that explained the most covariance were extracted to test for morph differences using *advanced.procD.lm*, followed by pairwise comparisons of morphs if significant. For those significant morph covariances, we examined the loadings on each PLS axis to determine the extent to which each gene had on shape. We tested for the affects of egg size on those overall significant covariances between relative gene expression and craniofacial shape using the first PLS axis in a Procrustes ANOVA whilst also accounting for morph differences (morph × egg size). Finally, we tested the effect of egg size within morphs using PLS1 based on any significant interactions between morphs and egg size.

5.4 Results

For analyses of gene expression, a total of 643 offspring were used across all morphs: 153 at PF, 161 at E, 161 at H and 168 at FF (Table 5.1). For shape analyses, a total of 494 offspring were used: 222 at H, and 272 at FF.

5.4.1 Gene expression differences within and among morphs and developmental stages

There was a significant morph × gene × dev. stage interaction ($F_{156,\ 10255} = 20.84$, P <0.0001; see Table S5.1) effect on gene expression. To disentangle these interaction effects, we next compared morph differences in gene expression by analysing the data within each developmental stage, and testing the interaction between morph × gene (Table 5.2), which was significant across all developmental stages (all P 0.001). Based on this significant interaction, we then examined morph differences in gene expression within each gene, where we found significant differences in the relative expression levels of most genes among morphs (see Table S5.2). Given the many and highly variable gene expression differences, we here only summarise the key points (Fig. 5.3) for each developmental stage.

Neither family nor cage had any effect on gene expression differences across development for VS and TLB, and only *Timp2* had a significant family effect for FJ ($X^2 = 6.24$, $P = 0.01$), whereas for TP family identity was significant for all genes (P <0.05) except for *Gr, Ghr1, Ctsk* and *Mmp9* (Table S5.3). At PF, *Ets2* and *Star* were not detected by qPCR for any morph except for TP, where they were both significantly expressed in comparison to zero ($P \leq .0001$); and FJ, where *Ets2* was detected in only three individuals (one from family 311, and two from family 308; Fig. 5.4). *Igf1* was also not detected at PF stage in VS and TLB (Fig. 5.3). *Mmp9* was detected at PF in all other morphs. However, VS only had one family (family 182) with offspring where *Mmp9* was detected (Fig. 5.4), and expression levels did not differ from zero. All other genes for all developmental stages differed significantly from zero ($P < 0.05$), for all morphs.

At PF, most genes were already expressed in all morphs but to variable extents (Fig. 5.3, Table S5.2). However, only TP showed substantial (relative) expression of the growth related *Star* and the skeletal related *Ets2* gene (both $P < 0.0001$; all other morphs showed either none or very low relative expression of these two genes) based on Tukey adjusted pairwise comparisons. *Igf1* was (relatively) expressed in only three (FJ, VB and TP) of the morphs. VS had higher relative expression of *Gr* and *Sparc* than the other morphs (all $P < 0.01$; Fig. 5.3). Relative expression levels of *Ghr1, Rictor* and *Igf2* (growth related) as well as *Sparc* and *Ctsk* (related to skeletogenesis) differed significantly among some of the morphs, whereas there were no morph differences in the relative expression of *Sgk1* and *Mtor* (related to growth), *Timp2, Mmp2* and *Mmp9* (related to skeletogenesis) at this stage (Fig. 5.3).

At E stage, growth related genes were relatively (in comparison to other developmental stages) highly expressed across all morphs. Strikingly however, VS had near two fold higher relative expression levels of all genes related to skeletogenesis than any other morph (all Tukey adjusted pairwise comparisons were $P < 0.0001$), whilst having relatively lower expression of the growth related *Star* and *Ghr1* (Fig. 5.3). At this stage, FJ had relatively higher expression of *Ghr1* than the other morphs (Tukey adjusted pairwise comparisons were all $P < 0.01$).

At H, all genes continued to be (relatively) highly expressed, and also showed variable expression levels among morphs. The most striking difference was that VS had nearly two fold higher expression of the growth related gene *Gr* ($P < 0.0001$, in all pairwise comparisons). Both VS and TP showed elevated expression of *Timp2* compared to other morphs at this stage ($P < 0.01$, for pairwise comparisons). *Igf1, Ghr1, Sparc* and *Mmp2* were not differentially expressed between any morphs at this stage (Fig. 5.3).

At FF, both VB and VS showed relatively lower expression of *Star* and *Sgk1* and elevated expression of *Igf2* compared to other morphs (Fig. 5.3). Both *Mtor* and *Rictor* showed very similar expression patterns across morphs, with FJ having lower expression of these genes than VB (Fig. 5.3). FJ also had relatively higher expression levels of *Sgk1* than the other morphs ($P < 0.0001$ for all pairwise comparisons). At this stage, VB differed most from the other morphs by showing relatively elevated expression of *Igf1*, *Mtor* and *Rictor* (growth related) as well as *Sparc, Ctsk, Etsk2* and *Mmp2* (related to skeletogenesis). Only two genes, *Gr* and *Ghr1,* were not differentially expressed between morphs at this stage.

Compared to the ancestral FJ population, weakly diverged morphs from lake Vatnshlíðarvatn had more genes that differed in relative expression than morphs from lake Þingvallavatn (N of genes that differed to FJ in Vatnshlíðarvatn/Þingvallavatn at: PF = 64% / 57%, E = 86% / 43%, H = 29% / 14%, FF = 86% / 29%; note that 14 genes were examined per developmental stage). As for the more derived morphs (VB and TLB), they were more similar in relative gene expression to the ancestral FJ than the less derived VS and TP, with more derived (VB and TLB) having 46% of genes that differed in relative expression levels to FJ, whereas less derived (VS and TP) had 55% that differed to FJ (Fig. 5.3). Nevertheless, the more derived morphs became more different in relative gene expression by the onset of feeding (79% of the genes differed, compared to 36% that differed in the less derived morphs). Finally, the number of genes that differed in sympatric morphs was also higher in the less diverged morphs from lake Vatnshlíðarvatn (32% of genes differed; at PF = 21%, E = 64%, H = 29%, and FF = 14%) compared to the more diverged morphs from lake Þingvallavatn (23% of genes differed; at PF = 36%, E = 36%, H = 21%, FF = 0%).

Taken together, relative gene expression levels showed clear differences among the five Arctic charr morphs, especially in terms of which mRNA transcripts are deposited in the egg by the mother. The differences between the more derived morphs (TLB and VB) and the putative ancestral population (FJ) were more numerous at first feeding than at earlier stages.

Table 5.2 *Results of mixed effect models testing for the effect of morph, gene identity (fixed factors) and their interaction on log (gene expression) across early development in the anadromous population and four morphs of Arctic charr (Salvelinus alpinus). Fish originated from the river Fljotáa (FJ), lake Vatnshlíðarvatn (brown and silver morphs), and Þingvallavatn (large benthic and planktivorous morphs). Relative gene expression of 14 genes (Beck et al., 2018) was characterised at four developmental stages: post-fertilisation (PF), eye stage (E), hatching stage, (H), and first feeding stage (FF). Family identity and cage (both nested within morph) were included as random effects the model.*

			Random Effects				Fixed Effects					
Dev. stage	N families	N	Variable	X^2	*df*	*P*	Variable	*Sum Sq*	*ndf*	*ddf*	*F*	*P*
PF	34	2559	**Family**	17.84	1	<.0001	Morph	**2.08**	4	45	1.78	0.159
			Cage	0.03	1	0.900	**Genes**	**274.72**	13	2461	72.51	<.0001
							Morph*Genes	**217.04**	52	2461	14.32	<.0001
E	34	2580	Family	0.00	1	1.000	**Morph**	**107.80**	4	46	50.24	<.0001
			Cage	19.80	1	<.0001	**Genes**	**312.95**	13	2461	44.87	<.0001
							Morph*Genes	**914.78**	52	2461	32.79	<.0001
H	34	2648	**Family**	11.10	1	<.0001	Morph	**1.66**	4	31	2.16	0.097
			Cage	30.70	1	<.0001	**Genes**	**26.21**	13	2526	10.47	<.0001
							Morph*Genes	**114.03**	52	2526	11.39	<.0001
FF	34	2809	**Family**	21.01	1	<.0001	**Morph**	**3.25**	4	29	2.98	0.035
			Cage	2.01	1	0.200	**Genes**	**46.82**	13	2696	13.22	<.0001
							Morph*Genes	**266.13**	52	2696	18.78	<.0001

Dev.stage; developmental stage. PF; post-fertilisation. E; eye stage. H; hatching stage. FF; first feeding stage. N families; total number of families. N; total number of observations in the model. X^2; Chi square statistic. *df*; number of degrees of freedom for the Chi square test. *P*; *P*-value of the likelihood ratio test for the random effect. *Sum Sq*; sum of squares. *ndf;* numerator degrees of freedom. *ddf;* denominator degrees of freedom based on Satterthwaite's approximations. *F*; F-value. Significant variables in bold. Response variable is log expression (log2, relative mRNA expression + 1).

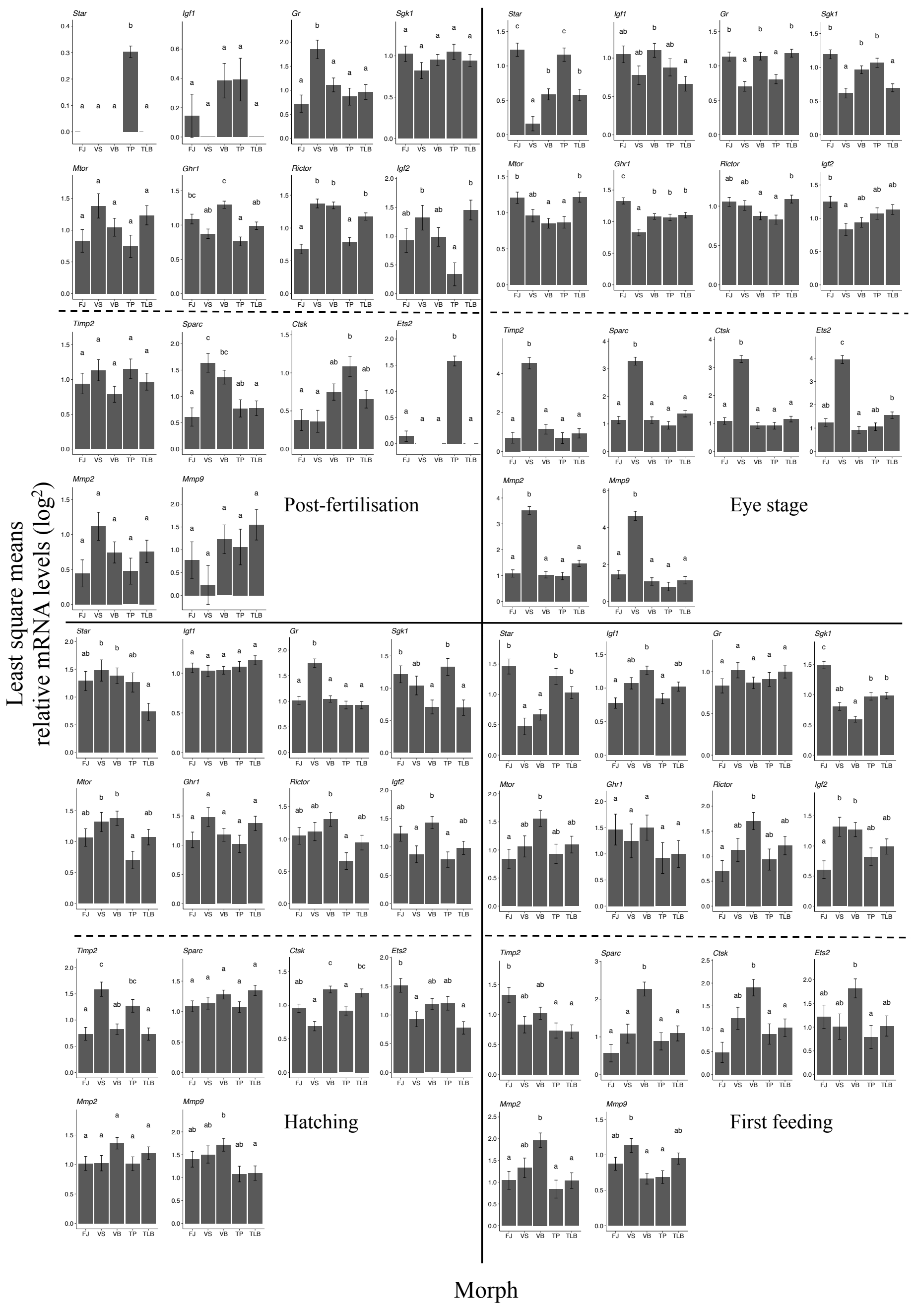


***Figure 5.3** Tukey adjusted pairwise comparisons of least-square (LS) means ± S.E. of relative mRNA levels of 14 genes in the anadromous population and four morphs of Arctic charr (Salvelinus alpinus) across early development (at post-fertilisation, eye stage, hatching and first feeding). Fish originated from river Fljótaá [FJ], lake Vatnshlíðarvatn silver [VS] and brown [VB] morphs, and lake Þingvallavatn planktivorous [TP] and large benthic [TLB]. Least-square means sharing a letter are not significantly different from each other. Solid lines show distinction between the developmental stages, whereas the*

dashed centerline separates the (above) growth related genes, and (below) genes involved in skeletogenesis within each developmental stage.

5.4.2 Individual size-correlated gene expression within morphs

We present here size-correlated gene expression results for each morph and developmental stage separately (Fig. 5.4, Table 5.3), based on significant differences between morphs and developmental stages (see above). Overall model fits and gene-specific model fits (i.e. whether a linear or polynomial model was more preferred) are presented in Table S5.5 and Table S5.6, respectively. The within morph data for VB is found in Beck et al. (2018) but we here present the figures and report the results where appropriate.

At PF, there was an overall significant effect of size on relative gene expression in all morphs except VB and TP (Table 5.3), but the significant gene × size interactions within all morphs indicated that the relationship between egg size and relative gene expression differed among genes (Table 5.3; Fig 5.4). Specifically, there was a negative relationship between size and gene expression for *Mmp9* for FJ (although small sample size, i.e. *Mmp9* was expressed only in a few individuals at PF for FJ, N = 8; Fig. 5.4a), a positive relationship in *Mmp9* for VS (N = 6, all of which came a single family; *Mmp9* was not detected in other VS families) and a non-linear relationship in *Sparc* for VS (Fig. 5.4c), and non-linear relationships in *Igf2* for TLB (Fig. 5.4d), as well as in *Ets2* and *Mmp2* for TP (Table 5.3; Fig. 5.4e). *Mmp9* was also correlated with egg size in VB at PF (Fig. 5.4b). When analysing the effect of size on individual genes using the best-fitting (linear or non-linear) model for each gene separately (Table S5.6), *Igf2* also correlated with size in VS (slope of *Igf2*: $t = 3.47$, $P = 0.008$; Table S5.7; Fig. 5.4c). Pairwise comparisons of slopes found expression levels of *Mmp9* and *Mmp2* to be higher in FJ, VB and VS, whilst *Ctsk* and *Ets2* had low relative expression in TLB and TP, respectively (Table S5.8). All morphs apart from FJ had a significant family effect at PF (all $P = \leq 0.01$; Table 5.3).

At E stage, there was a significant size × gene interaction in VB, VS and TP but no significant size effects on gene expression in FJ or TLB (Table 5.3b). *Post-hoc* comparison of slopes that differed from zero indicated that, in particular in VS, gene expression was correlated with egg size. In this morph *Rictor* and *Igf2* (related to growth) were weakly but significantly size-correlated in their expression levels, with *Rictor* having a positive correlation and *Igf2* being higher expressed in intermediate sized eggs (slope of both genes: $P < 0.05$; Table 5.3b; Fig 5.4c). Interestingly in VS all genes related to skeletogenesis showed a positive relationship with egg size (Fig. 5.4c and Table 5.3b). Although VS had numerous genes correlated with egg size, pairwise comparisons of slopes showed that relative gene expression differences did not differ between genes, except for *Sgk1* and *Igf2* where *Sgk1* had lower expression compared to *Igf2* (pairwise contrasts of slopes: $t = -3.78$, $P = 0.013$; Table S5.8). The significant interaction between size × gene (Table 5.3b and 5.3e) for VB and TP at E stage was likely due to relative expression differences between genes (as no gene was found to be strongly correlated with egg size and any relationship was likely lost after adjustment for multiple comparisons; Table 5.3b), primarily driven by *Mmp9* and *Mmp2* in VB, and *Mmp9* in TP having lower expression in comparison to pairwise contrasts (Table S5.8). At this stage, only VB had a significant family effect ($P < 0.0001$).

At H stage, all morphs again showed a significant size × gene interaction in gene expression (Table 5.3a-e), but the relationship between size at hatching and relative gene expression differed among morphs. In FJ, the expression of *Mmp9* was higher in relatively larger offspring (slope of *Mmp9*: $t = 6.00$, $P < 0.0001$). In VB, expression level of *Star* was non-linearly related to size at hatching whereas relative expression of *Sgk1* was higher in larger offspring (Fig. 5.4b; Beck at al., [2018], *Paper II*). In TLB, *Star* was more strongly expressed in smaller offspring and *Ghr1* in intermediate-sized offspring (Fig. 5.4d). Finally, in TP expression levels of *Star* and *Timp2* were relatively higher in larger offspring and *Sgk1* in intermediate-sized offspring (Fig. 5.4e). The only morphs to show a significant family effect on size-correlated relative gene expression levels at this stage were VB and TP ($P = \leq 0.01$).

At FF, there was no size effect in TLB, whereas the other three morphs showed significant size × gene interaction effects. In VB however, any significant relationship between size and relative gene expression was lost after adjustment for multiple comparisons. Pairwise comparisons of slopes found *Ghr1* to be higher expressed in comparison to *Gr* and *Timp2* in VB. *Igf2* was positively correlated with size in VS (slope of *Igf2* compared to zero: $t = 3.01$, $P = 0.038$; Fig. 5.4c), whilst relative expression of *Star, Sgk1, Ghr1* and (to some extent) *Mmp9* tended to increase with size at FF in TP (Fig. 5.4e). Similar to H, VB and TP were also the only morphs that had a significant family effect at FF ($P = <0.0001$)

Taken together, relative gene expression was frequently correlated with individual size, but the relationships were often non-linear and highly morph specific. The effect of family identity on relative gene expression also varied across morphs, with all morphs having a significant family effect at PF except for FJ, whilst VB has a significant family effect across all developmental stages. Most striking differences among morphs was seen in VS at eye stage, where the relative gene expression levels of all genes connected with skeletogenesis were positively related to egg size - something that was not seen in the other morphs (Table 5.3b). Genes most frequently associated in expression levels to size were *Star* and *Mmp9* (Fig. 5.4*)*. Only *Igf1, Gr* and *Mtor* (growth related) were never correlated with size in any of the morphs (including VB; Beck et al., 2018).

Table 5.3 *Results of best-fitted mixed effect models testing for the effect of gene identity (fixed factor) and individual size (covariate), as well as their interaction, on gene expression (see Table S5.7 for results using non-preferred mixed effects models) in Arctic charr from: a) Fljótaá, b) Vatnshlíðarvatn silver, c) Vatnshlíðarvatn brown, d) Þingvallavatn planktivorous and e) Þingvallavatn large benthic. These analyses were conducted separately for each developmental stage (PF, post-fertilisation; E, eye stage; H, hatching; FF, first feeding) due to significant differences among developmental stages (see Table S5.4). Family and cage were used as random effects. Data from VB are published in Beck et al. (2018; or Paper II of this thesis) but reported here to ease the comparison between the morphs.*

a)

Fljotáa (FJ)																	
			Random Effects				Fixed Effects										
Dev. stage	N	N ♀	**Variable**	X^2	*X* Df	P	Variable	Sum Sq	Num DF	Den DF	F	P	β	SE	Df	*t*	P
PF	350	6	Family	1.421	1	0.200	**Size**	2.23	1	144	8.60	**0.004**					
			Cage	0.271	1	0.600	**Genes**	6.05	13	312	1.79	**0.043**					
							Size*Genes	6.01	13	312	1.78	**0.045**					
							Mmp9						**1.39**	**0.29**	**320**	**4.76**	**<0.0001**
E	475	6	Family	0.889	1	0.300	Size	0.29	1	321	2.54	0.112					
			Cage	2.742	1	0.100	Genes	1.07	13	434	0.73	0.735					
							Size*Genes	1.09	13	434	0.74	0.725					
H	490	6	Family	1.57	1	0.210	Size	0.03	1	162	0.75	0.388					
			Cage	5.42	1	**0.020**	**Genes**	1.96	13	449	3.68	**<0.0001**					
							Size*Genes	2.32	13	449	4.35	**<0.0001**					
							Mmp9						**0.27**	**0.04**	**460**	**6.00**	**<0.0001**
FF	476	6	Family	0.324	1	0.600	**Size**	3.40	1	313	13.53	**<0.001**					
			Cage	2.469	1	0.100	Genes	1.64	13	440	0.50	0.923					
							Size*Genes	2.61	13	440	0.80	0.664					

b)

Vatnshlíðarvatn silver (VS)																	
Dev. stage	N	N ♀	Random Effects				Fixed Effects										
			Variable	X^2	*X* Df	*P*	Variable	Sum Sq	Num DF	Den DF	F	*P*	β	SE	Df	*t*	*P*
PF	418	5	**Family**	259.00	1	**<.0001**	**Size (poly^3)**	2.00	3	181	5.14	**0.002**					
							Genes	159.62	13	358	94.53	**<0.0001**					
							Size*Genes	14.82	39	358	2.93	**<0.0001**					
							Sparc						1.92	0.46	347	4.18	**0.001**
							Mmp9						-1.43	0.46	348	-3.12	**0.027**
E	416	5	Family	2.86	1	0.09	**Size**	174.05	1	323	92.12	**<.00001**					
			Cage	0.00	1	1	**Genes**	111.61	13	384	4.54	**<0.0001**					
							Size*Genes	146.4	13	384	5.96	**<0.0001**					
							Rictor						3.98	1.35	174	2.94	**0.050**
							Igf2						4.09	1.35	174	3.03	**0.039**
							Timp2						10.15	1.35	174	7.51	**<.0001**
							Sparc						7.19	1.35	174	5.32	**<.0001**
							Ctsk						7.24	1.35	175	5.36	**<.0001**
							Ets2						8.50	1.35	174	6.29	**<.0001**
							Mmp2						7.24	1.35	174	5.36	**<.0001**
							Mmp9						11.03	1.35	174	8.16	**<.0001**
H	396	5	Family	0.10	1	0.8	**Size (poly^2)**	11.075	2	63	10.22	**≤0.0001**					
			Cage	2.64	1	0.1	**Genes**	35.378	13	349	5.02	**<0.0001**					
							Size*Genes	24.425	26	349	1.73	**0.016**					
FF	418	5	Family	1.14	1	0.3	**Size**	1.9915	1	77	6.88	**0.010**					
			Cage	0.00	1	1	**Genes**	7.7756	13	386	2.07	**0.015**					
							Size*Genes	8.87	13	386	2.357	**0.005**					
							Igf2						**0.25**	**0.082**	**380.48**	**3.01**	**0.038**

c)

Vatnshlíðarvatn brown (VB)																	
Dev. stage	N	N ♀	Random Effects				Fixed Effects										
			Variable	X^2	*X* Df	*P*	Variable	Sum Sq	Num DF	Den DF	F	*P*	β	SE	Df	*t*	*P*
PF	546	7	**Family**	12.73	1	<.0001	Size (poly2)	1.85	2	6	4.56	0.064					
			Cage	0.05	1	0.827	**Genes**	94.22	13	489	35.71	**<0.0001**					
							Size*Genes	38.31	26	489	7.26	**<0.0001**					
							Mmp9						2.16	0.19	114	11.31	**<0.0001**
E	485	7	**Family**	14.60	1	<.0001	**Size (poly3)**	1.4	3	14	5.23	0.013					
			Cage	0.00	1	1	**Genes**	10.71	13	410	9.25	**<0.0001**					
							Size*Genes	11.16	39	410	3.21	**<0.0001**					
H	559	7	**Family**	9.04	1	0.003	Size (poly3)	0.22	3	85	0.37	0.778					
			Cage	6.84	1	0.009	**Genes**	37.73	13	488	14.27	**<0.0001**					
							Size*Genes	29.76	39	488	3.75	**<0.0001**					
							Star						0.69	0.14	432	5.02	**<0.0001**
FF	574	7	**Family**	16.65	1	<.0001	Size (poly2)	0.73	2	76	1.06	0.353					
			Cage	1.12	1	0.291	**Genes**	106.78	13	520	23.81	**<0.0001**					
							Size*Genes	27.3	26	520	3.04	**<0.0001**					

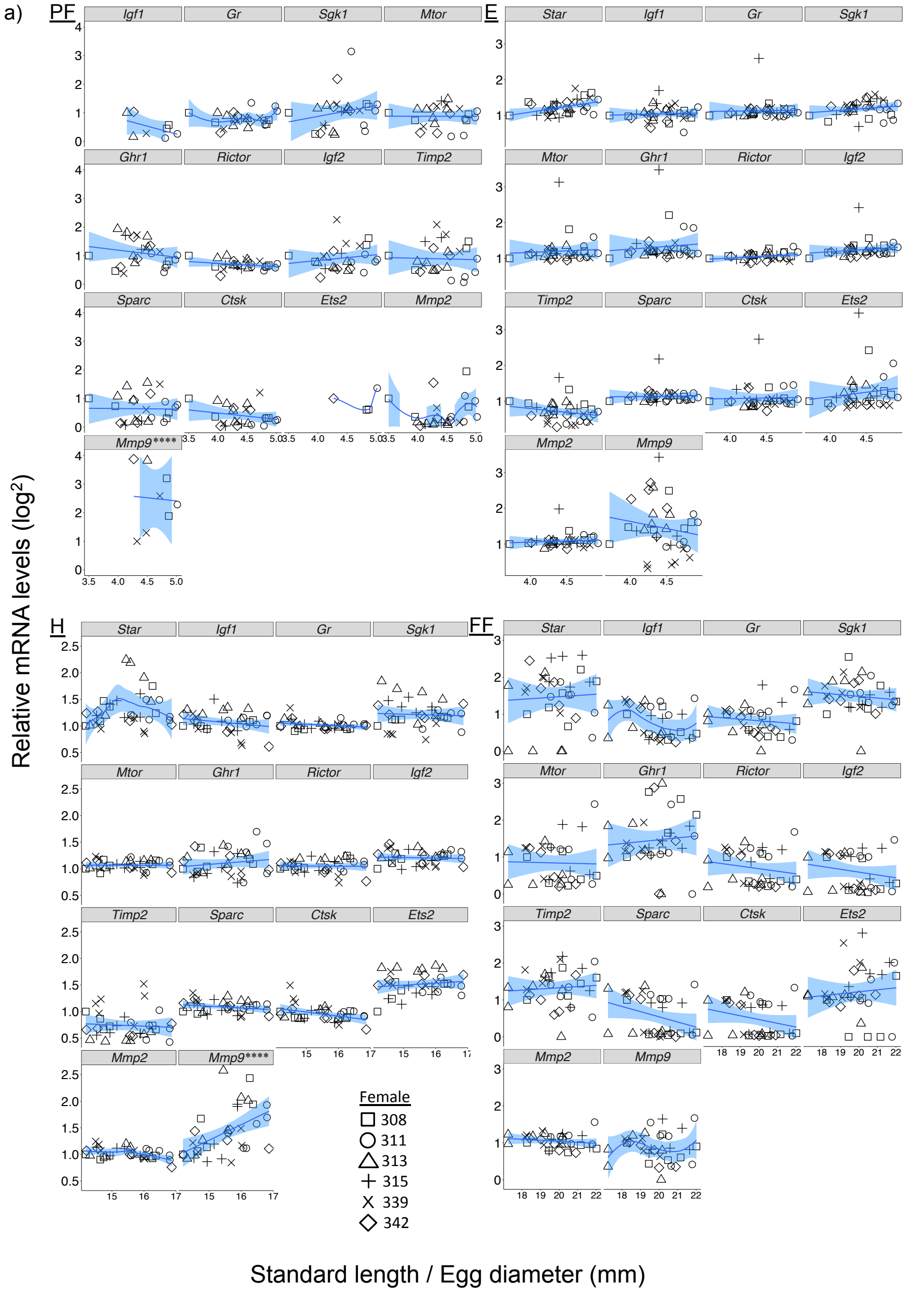
d)

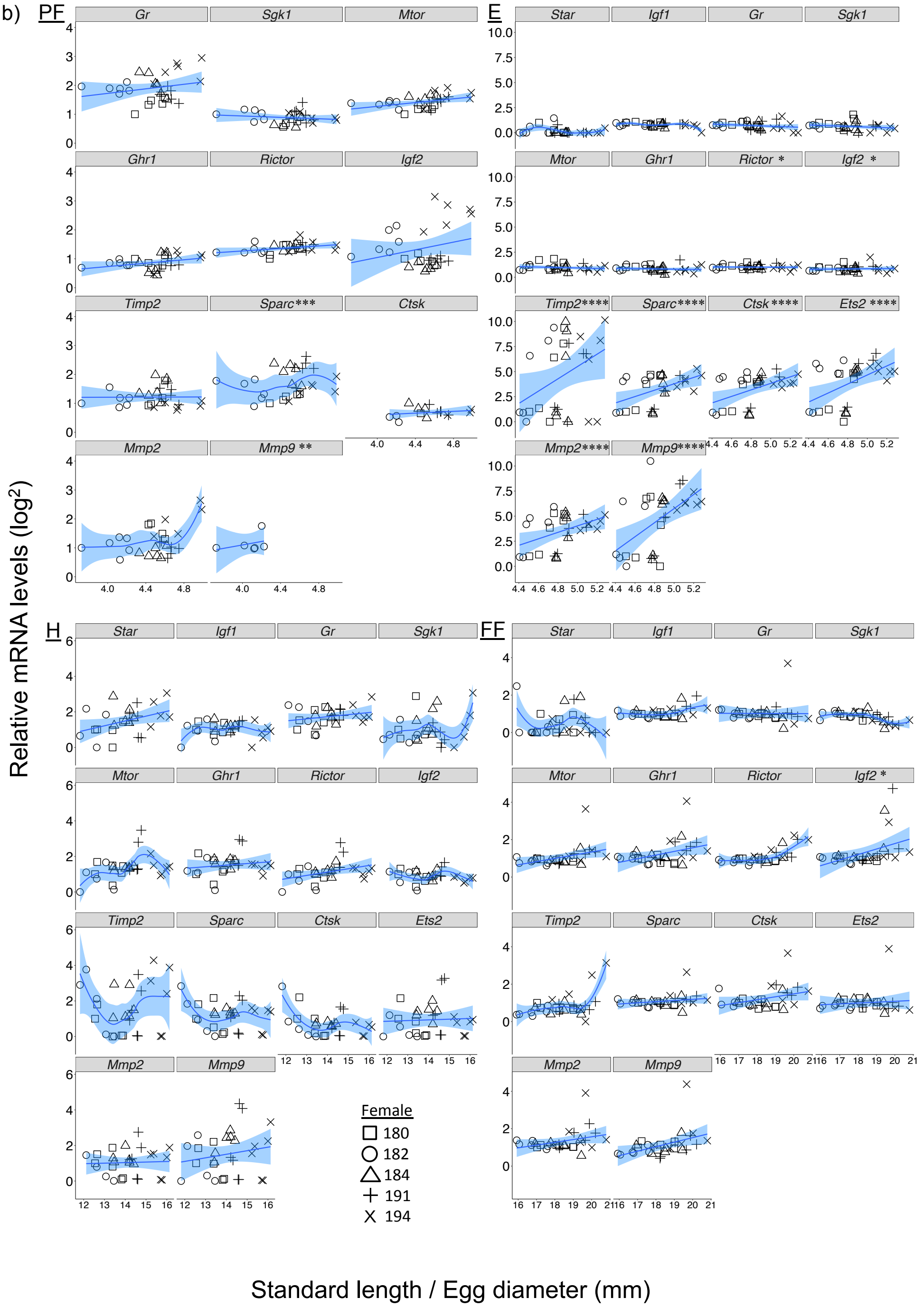
þingvallavatn planktivorous (TP)																	
			Random Effects				Fixed Effects										
Dev. stage	N	N ♀	Variable	X^2	*X* Df	*P*	Variable	Sum Sq	Num DF	Den DF	F	P	β	SE	Df	*t*	*P*
PF	447	6	**Family**	181.00	1	**<.0001**	Size (poly^2)	0.71	2	17	3.01	0.076					
							Genes	55.36	13	399	35.93	**<0.0001**					
							Size*Genes	14.11	26	399	4.58	**<0.0001**					
							Ets2						**-1.76**	**0.22**	**310**	**-7.96**	**<0.0001**
							Mmp2						**-0.66**	**0.22**	**310**	**-2.99**	**0.041**
E	462	6	Family	2.59	1	0.100	Size	0.01	1	12	0.71	0.417					
							Genes	0.52	13	430	2.03	**0.017**					
							Size*Genes	0.77	13	430	2.99	**<0.001**					
H	504	6	**Family**	10.80	1	**0.001**	Size	0.06	1	28	2.48	0.127					
							Genes	0.66	13	471	2.20	**0.009**					
							Size*Genes	0.89	13	471	2.97	**<0.001**					
							Star						**0.09**	**0.03**	**347**	**3.16**	**0.024**
							Timp2						**0.11**	**0.03**	**347**	**3.79**	**0.003**
FF	502	6	**Family**	104.00	1	**<.0001**	**Size (poly^2)**	0.29	2	25	7.44	**0.003**					
							Genes	9.16	13	453	36.61	**<0.0001**					
							Size*Genes	1.20	26	453	2.40	**0.0002**					
							Ghr1						**0.07**	**0.02**	**288**	**3.62**	**0.005**
							Sgk1						**0.06**	**0.02**	**288**	**3.04**	**0.036**
							Star						**0.11**	**0.02**	**287**	**5.39**	**<0.0001**

e)

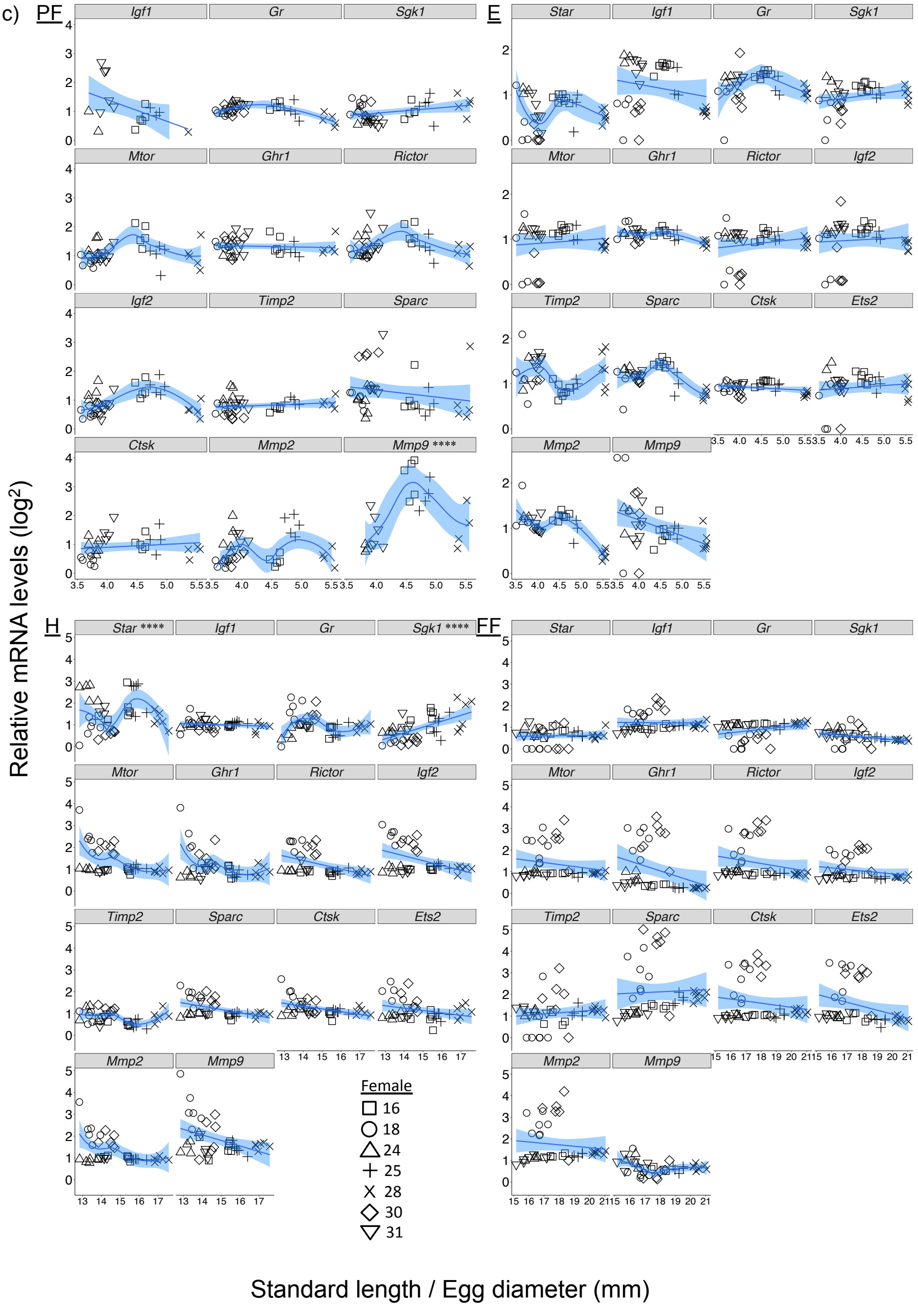
þingvallavatn large benthic (TLB)																
			Random Effects				Fixed Effects									
Dev. stage	N	N ♀	**Variable**	X^2	*X* Df	*P*	Variable	Sum Sq	Num DF	Den DF	F	*P*	β	SE	Df	*t*	P
PF	378	5	**Family**	6.26	1	**0.010**	**Size (poly^3)**	5.38	3	265	13.24	**<0.0001**					
			Cage	0.00	1	1.000	**Genes**	176.70	13	318	100.34	**<0.0001**					
							Size*Genes	19.19	39	318	3.63	**<0.0001**					
							Igf2						2.33	0.67	320	3.47	**0.008**
E	406	5	Family	0.00	1	1.000	Size	0.17	1	177	3.31	0.070					
			Cage	22.10	1	**0.000**	Genes	0.65	13	367	0.97	0.483					
							Size*Genes	0.80	13	367	1.18	0.289					
H	294	5	Family	0.22	1	0.600	Size	0.05	1	97	1.92	0.169					
			Cage	19.68	1	**0.000**	**Genes**	1.05	13	256	3.27	**≤0.0001**					
							Size*Genes	1.33	13	256	4.17	**<0.0001**					
							Star						-0.19	0.04	262	-4.30	**<0.001**
FF	391	5	Family	0.51	1	0.500	Size	0.06	1	22	1.37	0.255					
			Cage	0.00	1	1.000	Genes	0.28	13	359	0.50	0.927					
							Size*Genes	0.29	13	359	0.52	0.915					

Dev.stage; developmental stage. N; total number of observations used in the model. Log expression; log2 (relative mRNA expression+1). X^2; Chi square statistic. Chi. DF; number of degrees of freedom for the test. *P*; *P*-value of the likelihood ratio test for the random effect. Sum Sq; sum of squares. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Sattherthwaite's approximations. F; F-value. Gene slopes that remained significantly different from zero after Tukey's adjustments are indicated, as well as slope (β), standard error (SE), degrees of freedom (DF), *t*-ratio (*t*) and associated *P*-values. Significant variables in bold.


a)
PF
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9****
E
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
H
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9****
FF
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
Female
□ 308
○ 311
△ 313
+ 315
× 339
◇ 342
Relative mRNA levels (log²)
Standard length / Egg diameter (mm)


b)
PF
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc***
Ctsk
Mmp2
Mmp9 **
0
1
2
3
4
4.0
4.4
4.8
E
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor *
Igf2 *
Timp2****
Sparc****
Ctsk ****
Ets2 ****
Mmp2****
Mmp9****
0.0
2.5
5.0
7.5
10.0
4.4
4.6
4.8
5.0
5.2
H
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
0
2
4
6
12
13
14
15
16
FF
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2 *
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
0
2
4
16
17
18
19
20
21
Relative mRNA levels (log²)
Female
□ 180
○ 182
△ 184
+ 191
X 194
Standard length / Egg diameter (mm)

c)


PF
Igf1
Gr
Sgk1
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Ghr1
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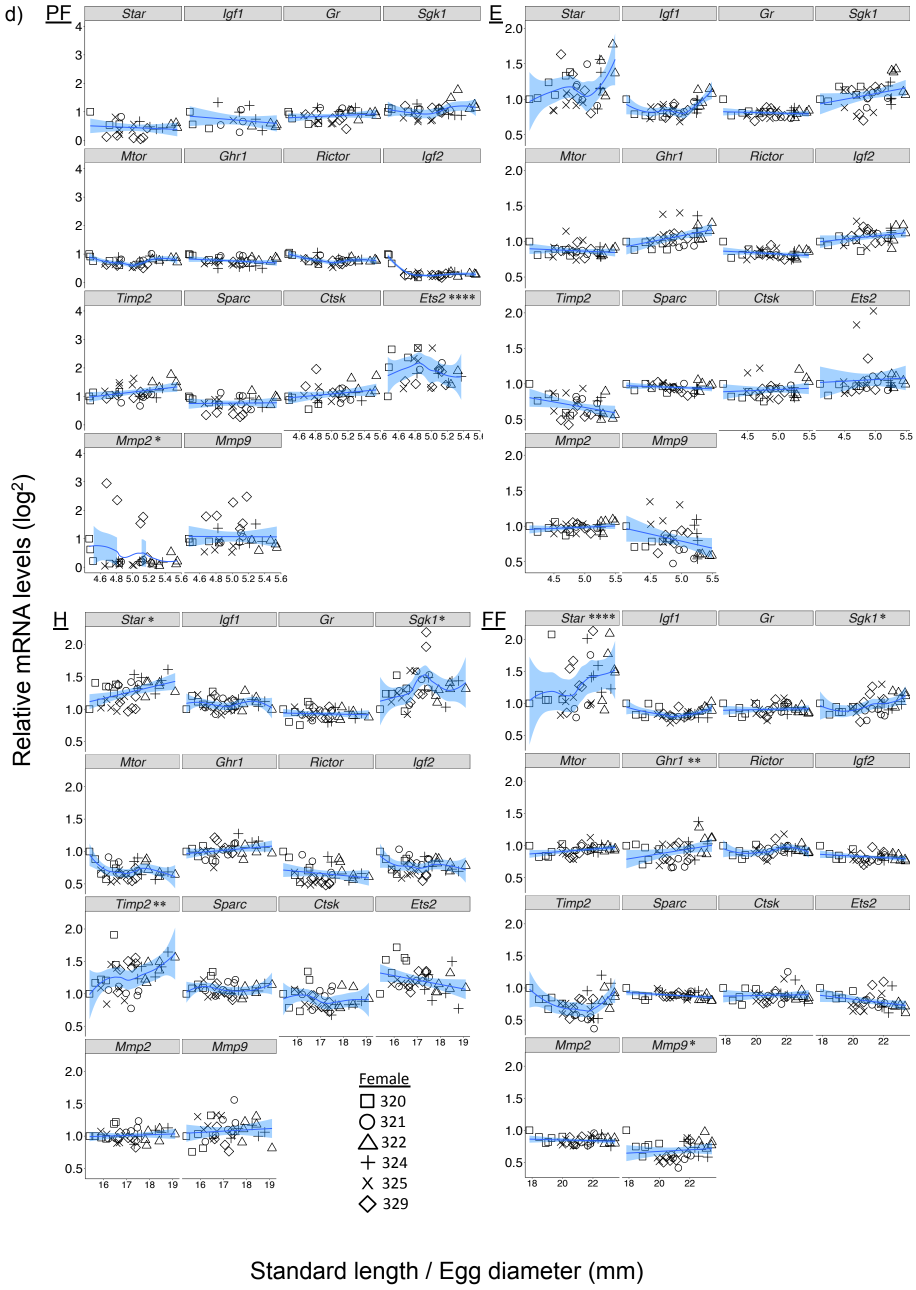
Timp2
Sparc
Ctsk
Mmp2
Mmp9 ****
E
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
H
Star ****
Igf1
Gr
Sgk1 ****
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
FF
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2
Mmp2
Mmp9
Relative mRNA levels (log²)
Standard length / Egg diameter (mm)
3.5 4.0 4.5 5.0 5.5
13 14 15 16 17
15 16 17 18 19 20 21
Female
□ 16
○ 18
△ 24
+ 25
× 28
◇ 30
▽ 31


d)
PF
E
H
FF
Star
Igf1
Gr
Sgk1
Mtor
Ghr1
Rictor
Igf2
Timp2
Sparc
Ctsk
Ets2 ****
Mmp2 *
Mmp9
Star *
Sgk1*
Timp2 **
Star ****
Sgk1 *
Ghr1 **
Mmp9 *
Relative mRNA levels (log2)
Female
□ 320
○ 321
△ 322
+ 324
× 325
◇ 329
Standard length / Egg diameter (mm)

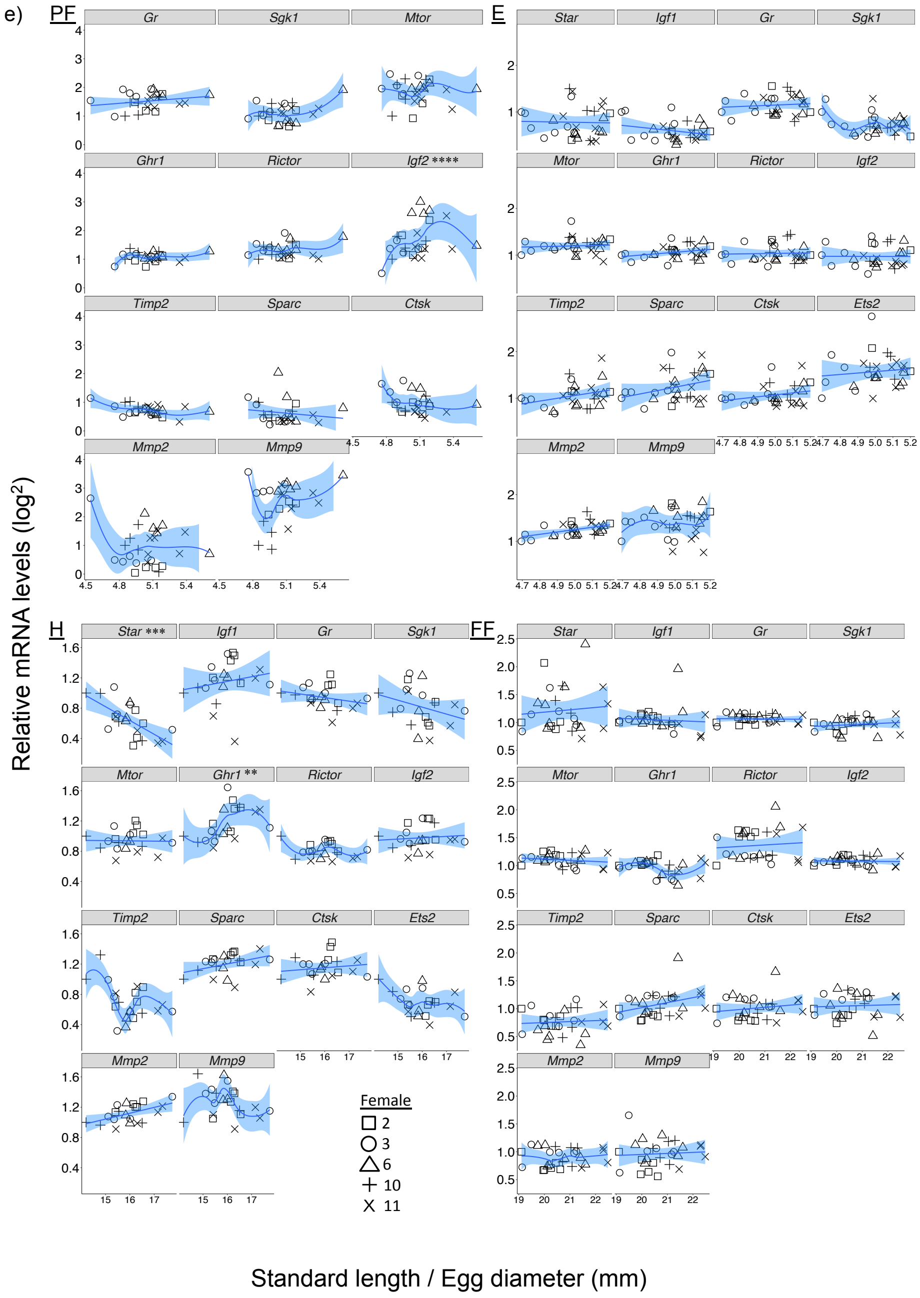


Figure 5.4 *Relative mRNA levels and their correlation with egg/individual size at: post-fertilisation (PF), eye stage (E), hatching (H) and first feeding (FF) in Arctic charr (Salvelinus alpinus) from (a) Fljótaá (FJ), (b) Vatnshlíðarvatn silver (VS), (c) Vatnshlíðarvatn brown (VB; modified from Beck et al., 2018), (d) Þingvallavatn planktivorous (TP), and (e) Þingvallavatn large benthic (TLB). Note that at PF, the following genes were not detected by qPCR and have been removed from the figure in*

*some morphs: Star was not detected for all morphs except TP; Ets2 was not detected in FJ or TLB; and Igf1 was not detected in VS or TLB). Both linear and non-linear fits were tested initially for each gene (see Methods section) and the results of the best fitting model represented here. Genes that are significantly correlated with size are indicated in the gene names of the plots by: P *< 0.01, **<0.001 and ***<0.0001. Symbols correspond to different family identities (i.e. different clutches).*

5.4.3 Egg size and craniofacial shape differences between morphs

Morph identity had a significant effect on craniofacial shape both before ($Z_{4,\,53}$ = 2.80, *P* = 0.004) and after ($Z_{4,\,53}$ = 2.23, *P* = 0.018) accounting for differences due to egg size (i.e. allometry). Developmental stage was also significant both before ($Z_{1,\,53}$ = 2.52, *P* = 0.037) and after accounting for egg size ($Z_{4,\,53}$ = 2.65, *P* = 0.032). Based on the significant differences in shape between developmental stages (Table 5.4), all further analyses were conducted within each developmental stage. Results from Procrustes ANOVA found craniofacial shape to differ significantly among morphs at H before accounting for differences in egg size ($Z_{4,\,26}$ = 2.24, *P* = 0.011), but morph differences in shape were lost after egg size was included in the model ($Z_{4,\,26}$ = 1.57, *P* = 0.087). However, egg size did not have an effect on craniofacial shape at hatching ($Z_{1,\,26}$ = 0.92, *P* = 0.239). For the significant morph effect before accounting for egg size, *post-hoc* pairwise comparisons showed that craniofacial shape differed significantly between the anadromous FJ population and the pelagic TP morph ($Z_{4,\,26}$ = 2.19, *P* = 0.015). Sympatrically, although weak (based on effect size, Z), only VB and VS morphs differed in craniofacial shape at H (pairwise comparisons: $Z_{4,\,26}$ = 1.91, *P* = 0.039). However, TLB had a low sample size (total N families = 3) and tests may therefore lack power to detect differences in shape at this early stage of development. Visual assessment of deformation grids indicated that FJ had a wide hyoid arch (similar to VB) compared to all other morphs (Fig. 5.5). These differences in shape do not remain after accounting for allometry (see above).

At FF, morphs had a weak effect on craniofacial shape before accounting for differences due to egg size ($Z_{4,\,26}$ = 1.78, *P* = 0.052), which became stronger after accounting for this ($Z_{4,\,26}$ = 1.87, *P* = 0.044). However, egg size has no effect on craniofacial shape ($Z_{1,\,26}$ = 1.02, *P* = 0.201). *Post-hoc* pairwise comparisons of the significant morph effect showed shape differences between the sympatric TLB and TP morphs both before ($Z_{4,\,26}$ = 2.12, *P* = 0.021) and after ($Z_{4,\,26}$ = 2.05, *P* = 0.026) accounting for differences due to offspring size with the TLB morph having a much wider hyoid arch. No other planned pairwise comparisons were significant.

Based on significant differences between morphs, we examined whether egg size might have an effect on any craniofacial shape differences within morphs. Only VS at first feeding was found to have significant differences in shape between offspring from small and large eggs (Procrustes ANOVA: $Z_{4,\,1}$ = 2.03, *P* = 0.012; Fig. 5.6), with offspring from larger eggs starting to feed with a more bluntened snout than those smaller conspecifics, which show a contraction in shape at the terminal end of the Meckel’s cartilage (Fig. 5.6).

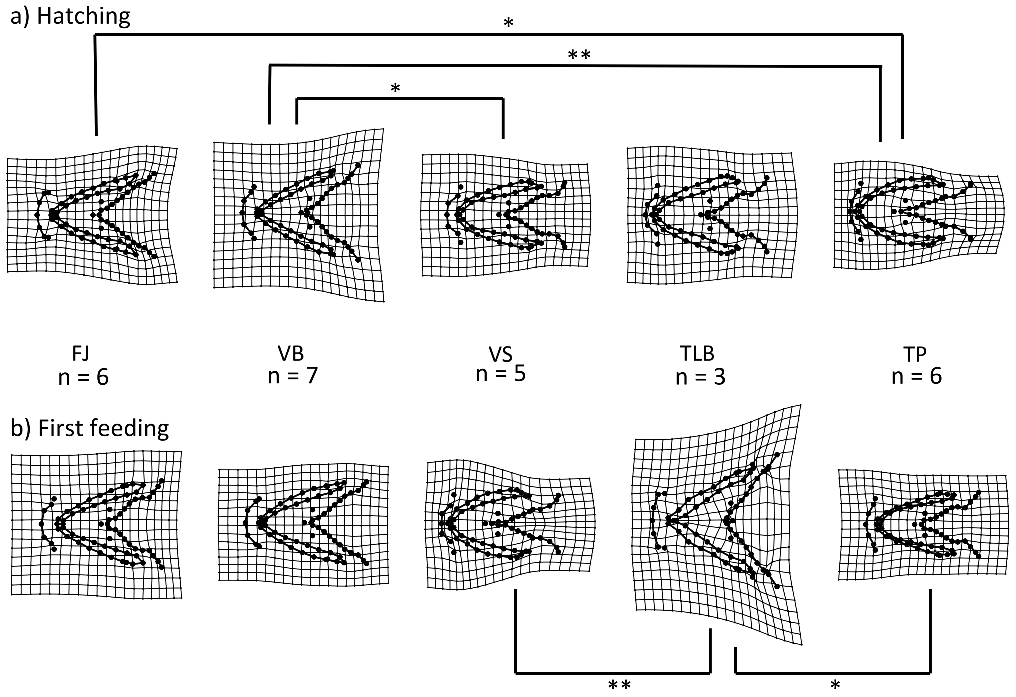


***Figure* 5.5** *Deformation grids showing mean ventral craniofacial morphology (using 17 fixed 54 semi-landmarks) in the anadromous population and four morphs of Arctic charr (Salvelinus alpinus) in Iceland, relative to overall mean morphology (before accounting for differences due to allometry) at a) hatching and b) first feeding. The five morphs were FJ, Fljótaá, VB, Vatnshlíðarvatn brown, VS, Vatnshlíðarvatn silver, TLB, Þingvallavatn large benthic and TP, Þingvallavatn planktivorous. Sample sizes (n) indicate number of families per morph (minimum N of individuals per family = 4; Table 5.1). Deformation grids are magnified by 4. After accounting for allometry at hatching (size differences), no morph differences in craniofacial shape remain ($Z_{4,\ 21} = 1.57$, $P = 0.087$), whereas TLB remains significantly different from VS ($Z_{4,\ 26} = 2.66$, $P = 0.002$) and TP ($Z_{4,\ 26} = 2.05$, $P = 0.026$) after accounting for allometry at first feeding (Table 5.4). Significance of pairwise post-hoc comparisons from the model without accounting for differences in allometry: $P <0.05^{*}$ $<0.01^{**}$ $<0.001^{***}$.*

Table 5.4 *Results from Procrustes ANOVA examining how craniofacial shape varies in the anadromous population and four morphs of Arctic charr (FJ, Fljótaá, VB, Vatnshlíðarvatn brown, VS, Vatnshlíðarvatn silver, TLB, Þingvallavatn large benthic and TP, Þingvallavatn planktivorous) due to: a) across morphs and developmental stages; b) across morphs and developmental stages accounting for mean egg size (per family); c) across morphs at hatching; d) across morphs at hatching accounting for mean egg size (per family); e) across morphs at first feeding; f) across morphs at first feeding accounting for the effects of mean egg size (per family); g) with mean egg size (per family) in Vatnshlíðarvatn silver; h) with morph and mean egg size (per family) on the covariance between craniofacial shape and skeletal related (relative) gene expression (using partial least squares component 1, PLS1) at hatching, and i) morph and mean egg size (per family) on the covariance between craniofacial shape and growth related (relative) expression of genes at first feeding.*

a) Across morphs and developmental stages

	Df	SS	R^2	F	Z	*P*
Morph	4	0.02	0.23	3.84	2.80	**0.004**
Dev.stage	1	0.01	0.06	3.80	2.52	**0.037**
Residuals	48	0.07				

b) Effects of egg size on shape across morphs and developmental stages

	Df	SS	Rsq	F	Z	*P*
Morph	4	0.02	0.18	3.06	2.23	**0.018**
Dev.stage	1	0.01	0.06	3.91	2.65	**0.032**
Egg size	1	0.01	0.08	5.81	2.91	**0.024**
Residuals	47	0.07				

c) Across morphs at hatching

	Df	SS	R^2	F	Z	*P*
Morph	4	0.01	0.39	3.48	2.24	**0.011**
Residuals	22	0.02				

d) Effects of egg size on shape across morphs at hatching

	Df	SS	R^2	F	Z	*P*
Morph	4	0.01	0.21	1.93	1.57	0.087
Egg size	1	0.00	0.03	1.17	0.92	0.239
Residuals	21	0.02				

e) Across morphs at first feeding

	Df	SS	R^2	F	Z	*P*
Morph	4	0.02	0.31	2.52	1.78	0.052
Residuals	22	0.04				

f) Effects of egg size on shape across morphs at first feeding

	Df	SS	R^2	F	Z	*P*
Morph	4	0.02	0.32	2.59	1.87	**0.044**
Egg size	1	0.00	0.04	1.34	1.02	0.201
Residuals	21	0.04				

g) Effects of egg size on shape in Vatnshlíðarvatn silver

	Df	SS	R^2	F	Z	*P*
Egg size	1	0.00	0.58	4.11	2.03	**0.012**
Residuals	3	0.00				

h) Effects of morph and egg size on the covariance between shape and skeletal related gene expression at hatching

	Df	SS	R^2	F	Z	*P*
Morph	3	0.00	0.55	7.86	3.39	**<0.001**
Egg size	1	0.00	0.00	0.13	0.10	0.703
Residuals	19	0.00				

i) Effects of morph and egg size on the covariance between shape and growth related gene expression at first feeding

	Df	SS	R^2	F	Z	*P*
Morph	3	0.01	0.47	12.84	2.96	**0.004**
Egg size	1	0.00	0.01	1.16	0.37	0.439
Morph*Egg size	3	0.01	0.32	8.60	3.89	**≤0.0001**
Residuals	16	0.00				

Dev.stage; developmental stage. SS; sum of squares. DF; degrees of freedom. F; F-value. Z; effect size. *P; P*-value to determine the significance of fixed effects.

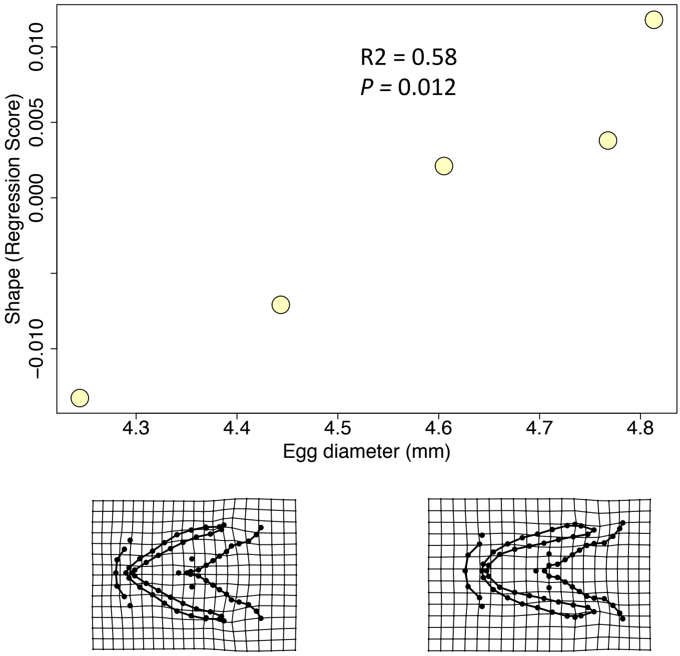
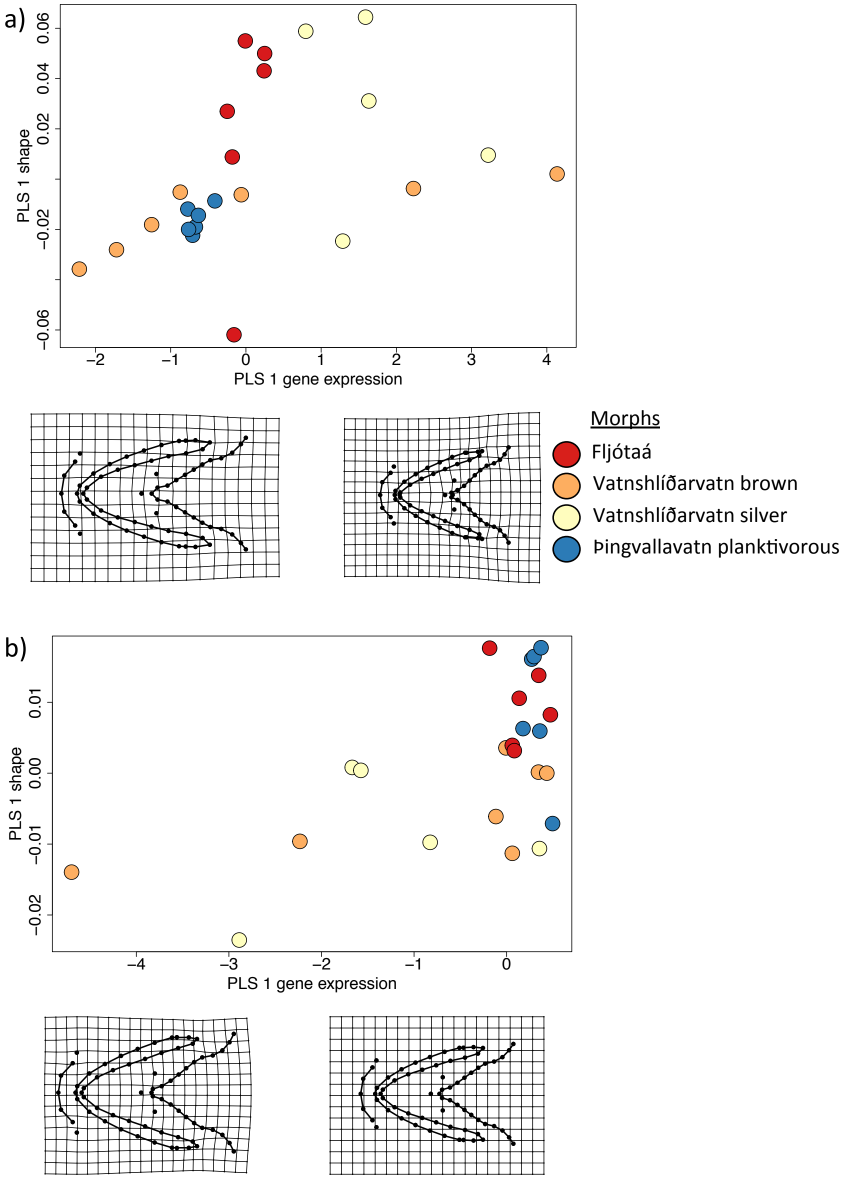


***Figure 5.6** Relationship between mean egg size (diameter) per family and craniofacial morphology in the Vatnshlíðarvatn silver Arctic charr morph at first feeding. Analysis is a Procrustes regression to evaluate egg size covariation with shape. Each point represents family means (N family = 5; N offspring per family = >10). Deformation grids show shapes associated with the smallest and largest egg sizes, magnified x 4.*

5.4.4 Relationship between gene expression and craniofacial shape

When conducting PLS analysis at each developmental stage we found significant overall covariance between the relative expression of: a) skeletal related genes and head shape at H (r_{PLS} = 0.58, P = 0.023; PLS 1 = 65% covariance; Fig. 5.7b), where there were only negative loadings, the highest of which came from *Mmp9* [-0.70], and b) growth related genes at FF (r-PLS = 0.67, P = ≤0.01; PLS 1 = 91% covariance; Fig. 5.7c), with the highest positive and negative loadings coming from *Igf2* [0.61] and *Star* [-0.29]. All covariance matrices between relative gene expression and craniofacial shape differed among morphs (*P* <0.05; Table S5.9). Pairwise comparisons of morphs revealed that the covariance between gene expression and craniofacial shape of VS differed consistently from those of other morphs. The only exception was for skeletal related gene expression at H, which found no difference in the covariance of relative expression and shape between the sympatric VS and VB morphs, but both of these morphs were significantly different in comparison to FJ and TP (Table 5.5). A significant interaction between morph and egg size was only found in the covariance between craniofacial shape and growth related gene expression at FF ($Z_{3,\,16}$ = 3.90, *P* <0.0001), yet further examination revealed no significant differences between morphs in egg size related changes in covariance (Table S5.10).


a)
PLS 1 shape
0.06
0.04
0.02
−0.02
−0.06
−2
−1
0
1
2
3
4
PLS 1 gene expression
Morphs
Fljótaá
Vatnshlíðarvatn brown
Vatnshlíðarvatn silver
Þingvallavatn planktivorous
b)
PLS 1 shape
0.01
0.00
−0.01
−0.02
−4
−3
−2
−1
0
PLS 1 gene expression

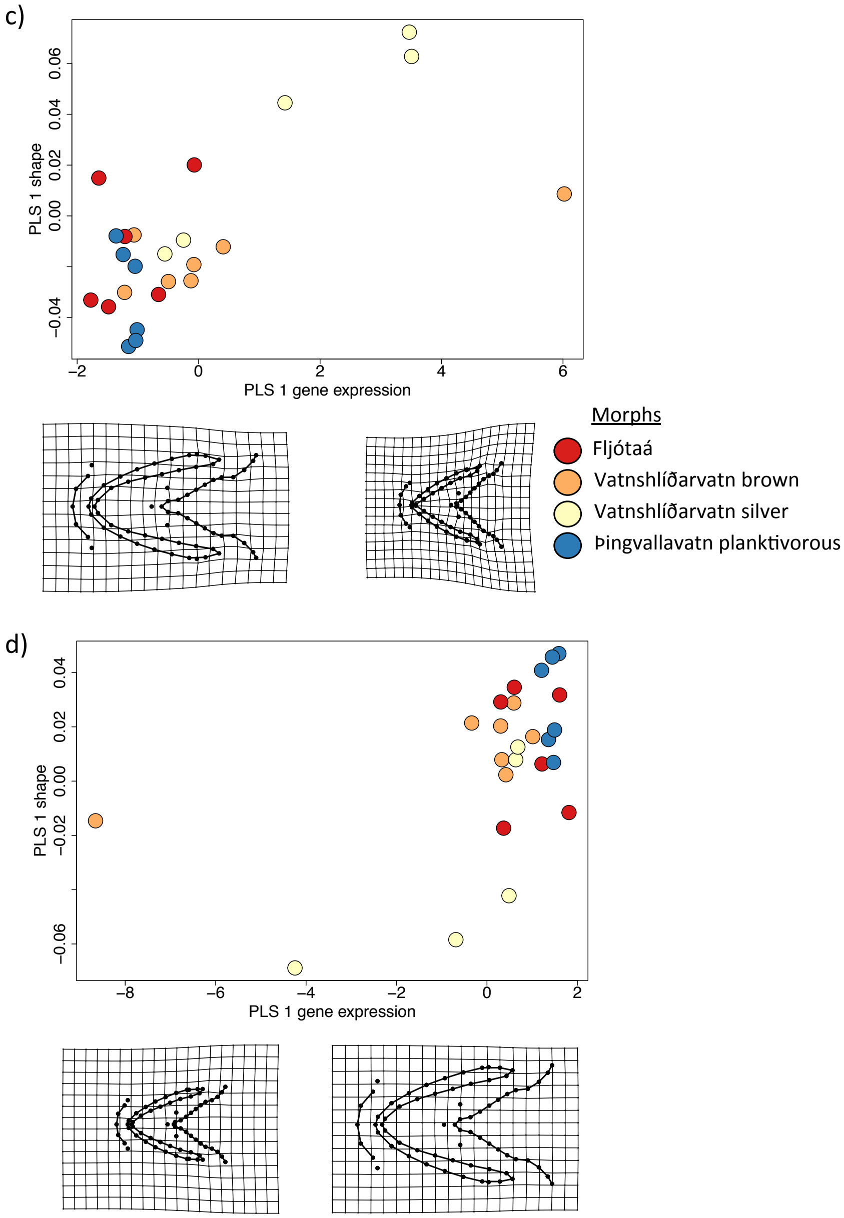


Figure 5.7 *Partial least square (PLS) analyses of covariance between relative gene expression and craniofacial shape in the anadromous population and three morphs of Arctic charr (FJ, Fljótaá, VS, Vatnshlíðarvatn silver, VB, Vatnshlíðarvatn brown and TP, Þingvallavatn planktivorous) for: a) growth related genes at hatching stage (r-PLS 0.38, P = NS; PLS1 = 73% covariance); b) genes involved in skeletogenesis at hatching (r-PLS = 0.58, P = 0.023; PLS 1 = 65% covariance); c) growth related genes at first feeding (r-PLS = 0.67, P = ≤0.01; PLS 1 = 91% covariance); and d) genes involved in skeletogenesis at FF (r-PLS = 0.49, P = NS; PLS1 = 89% covariance). Deformation grids show shapes*

associated with the extreme values of PLS1 relative to overall shape at that particular developmental stage, with plot (a) magnified by 2, (b) magnified by 0, (c) magnified by 2, and (d) magnified by 0. All PLS1 have significant morph effects (includes the anadromous population), see Table 5.5 for post-hoc pairwise comparisons of group means where this effect is mainly driven by Vatnshlíðarvatn silver. The points represent family means.

Table 5.5 *Pairwise comparisons of covariances between craniofacial shape and relative gene expression in the anadromous population and three morphs of Arctic charr (Salvelinus alpinus from FJ, Fljótaá, VB, Vatnshlíðarvatn brown, VS, Vatnshlíðarvatn silver and TP, Þingvallavatn planktivorous). For a) growth related genes at hatching, b) skeletal related genes at hatching, c) growth related genes at first feeding, and d) skeletal related genes at first feeding Effect sizes (Z) in the bottom half of matrices and associated P values above.*

a) Growth related genes at hatching
(r-PLS 0.38, P = NS; PLS1 = 73% covariance)

	FJ	TP	VB	VS
FJ	-	0.054	0.064	0.714
TP	1.907	-	0.895	**0.025**
VB	1.851	0.135	-	**0.027**
VS	0.378	**2.198**	**2.150**	-

b) Skeletal related genes at hatching
(r-PLS 0.58, P = 0.023; PLS 1 = 65% covariance)

	FJ	TP	VB	VS
FJ	-	0.961	**0.013**	**0.004**
TP	0.052	-	**0.017**	**0.006**
VB	**2.405**	**2.342**	-	0.628
VS	**2.693**	**2.607**	0.488	-

c) Growth related genes at first feeding
(r-PLS = 0.67, P = ≤0.01; PLS 1 = 91% covariance)

	FJ	TP	VB	VS
FJ	-	0.322	0.838	**0.025**
TP	1.024	-	0.402	**0.000**
VB	0.212	0.861	-	**0.010**
VS	**2.198**	**3.179**	**2.476**	-

d) Skeletal related genes at first feeding
FF (r-PLS = 0.49, P = NS; PLS1 = 89% covariance)

	FJ	TP	VB	VS
FJ	0.000	0.350	0.984	**0.020**
TP	0.964	0.000	0.314	**0.000**
VB	0.022	1.031	0.000	**0.014**
VS	**2.278**	**3.203**	**2.341**	0.000

5.5 Discussion

Our results on comparisons of multiple Arctic charr morphs originating from different populations demonstrate very dynamic patterns of gene expression across early life-stages, as well as craniofacial shape differences among morphs (similar to Kapralova et al., 2015). We also highlight the potential for maternal effects (egg size) for influencing differences in gene expression and craniofacial shape. Moreover, the specific patterns of covariation among the (relative) expression of genes related to growth and skeletogenesis and craniofacial morphology differed among morphs. Collectively, our results provide insight into the complex interplay of processes underlying the evolution of resource polymorphism in Arctic charr (*Salvelinus alpinus*).

5.5.1 Evidence for egg size-mediated maternal effects on gene expression

Gene expression before the mid-blastula transition is expected to reflect primarily maternally transmitted RNA (Kane & Kimmel, 1993). This transition to zygotic gene expression occurs at 42 degree days in Atlantic salmon (*Salmo salar;* Nagasawa et al., 2013). In our previous study on the brown morph from lake Vatnshlíðarvatn (VB here), we found no transcripts at the post-fertilisation (PF) stage for *Ets2* and *Star* (Beck et al., 2018). In the current study, the same pattern for both genes was found for the silver morph from lake Vatnshlíðarvatn (VS) and the large benthic morph from lake þingvallavatn (TLB; Fig. 5.3), whereas offspring from two families showed some expression of *Ets2* in the anadromous Fljótaá (FJ) population. In contrast, both genes were highly expressed in the planktivorous morph from þingvallavatn (TP). Moreover, *Igf1* (a growth factor) was only detected in two families in VB (Beck et al., 2018) and was in the current study not detected at the PF stage in VS or TLB. A previous study on developing Arctic charr morphs from lake þingvallavatn found that *Ets2* was relatively more expressed in benthic compared to pelagic morphs at all developmental stages except hatching (Ahi et al., 2014). In our study here, we also found similar expression patterns, with *Ets2* having no relative expression differences between the two þingvallavatn morphs at hatching stage. The pelagic planktivorous TP morph did, however, show relatively higher expression of *Ets2* at post-fertilisation than the benthic morph of lake þingvallavatn (Fig. 5.3), a developmental stage that was not sampled by Ahi et al. (2014).

Our results suggest clear differences in maternal mRNA deposition among morphs as well as between families of the same morph (Table 5.3), including the anadromous population. Such variation in maternally deposited mRNA promotes the development of variable individuals that may be important for early divergence in developmental processes (Gilbert & Epel, 2009). In our study, *Ets2* at post-fertilisation was correlated with egg size in the TP morph, with embryos from intermediate-sized eggs having higher expression (Fig. 5.4). As *Ets2* is a potential regulator of a gene expression network associated with craniofacial divergence in Arctic charr (Ahi et al., 2014). Given its potential for egg size-correlated expression at post-fertilisation, this transcription factor may provide a mechanism for maternally-mediated early craniofacial divergence in Arctic charr (Beck et al., 2018).

Consistent with patterns of gene expression in the VB morph from lake Vatnshlíðarvatn (Beck et al., 2018), we found that relative expression levels of *Mmp9* at PF was correlated with individual egg size in both the FJ and VS morphs (Fig. 5.4). However, because only a small number of offspring (N = 8) had detectable transcripts of this gene in FJ, and also in VS where transcripts of *Mmp9* were only detected in offspring from one family (Fig. 5.4), inferences regarding the egg size-correlated pre-zygotic (relative) expression of *Mmp9* in more weakly diverged morphs can not yet be made.

Based on previous findings that showed smaller offspring from smaller eggs i) start feeding essentially at the bottom (Leblanc et al., 2011), and ii) direct more energy towards ossification (Eiríksson et al., 1999) in comparison to their larger conspecifics, we predicted that smaller eggs would have higher (relative) expression of genes involved in skeletogenesis. However, in contrast, in VS we found all genes related to skeletogenesis to have higher relative expression in larger eggs (Fig. 5.4), and these genes also had almost two fold higher relative expression compared to other morphs, including the anadromous population (Fig. 5.3). Before hatching, the embryo continues to form cartilaginous structures to prepare for transition to the external environment. A recent study on lake Malawi cichlid fish documents how movement (i.e. gaping frequency) can induce a shift in skeletal development, with associated differences in *ptch1*, a gene involved in interspecific skeletal shape differences (Roberts et al., 2011; Hu & Albertson, 2017). Although the aforementioned study is conducted on post-hatching offspring, active movement from embryos within the confines of the egg has been associated with the correct development of cartilage and bone in other species, such as chickens (Müller, 2003). The strong correlation between egg size and the relative expression of genes related to skeletogenesis in VS suggests that developmentally plastic responses of offspring, such as ossification rates, are altered in response to egg size. Alternatively, one can consider the influence that the spawning strategy of mothers can have on newly hatched offspring. Similarly to the anadromous population, VS migrate up-stream to spawn. The rate of ossification could reflect the functional needs of offspring (Mabee et al., 2000; Harrington et al., 2013), with rates in post-hatching Arctic charr differing among populations inhabiting different water velocities (Cloutier et al., 2010). The anadromous FJ population, however, does not share such egg size-correlated gene expression patterns with VS at either eye stage, hatching or first feeding (Fig. 5.4).

We predicted that the more derived morphs would be less similar in relative gene expression in comparison to the putative ancestral FJ population. Across all developmental stages, we found that this prediction does not hold with the less derived (VS and TP) morphs having a higher number of genes differentially expressed in comparison to FJ (55%) than the more derived morphs (VB and TLB; 46%). However, when examining these patterns more closely (i.e. within developmental stages), the pattern reverses by the onset of first feeding with the more derived morphs having 79% of genes differentially expressed to FJ in comparison to the less derived morphs with 35% dissimilarity. This finding therefore lends some support to our prediction, albeit only at first feeding stage. Such higher number of genes differentially expressed in less derived morphs compared to FJ might be reflective of the specialisation of trophic structures required for first feeding. The diet of anadromous charr can be very variable depending upon fish size, maturity, environmental conditions, and availability of prey (Dempson et al., 2002). Another study examining a single population of anadromous charr found that the diet of adult fish consisted more of pelagic organisms, such as *Holopedium* sp. and chironomid pupae (Rikardsen et al., 2003). Nevertheless, benthic invertebrates were also found in the diets of anadromous charr in both aforementioned studies. The lack of such differential expression of more derived morphs with FJ in earlier developmental stages in comparison to the less derived morphs might suggest that either: 1) the original developmental variants that facilitated the more specialised feeding behaviours of the more derived morphs have been lost as trophic structures become more canalised (Parsons et al., 2010), and/or 2) the expression of genes important for growth and skeletogenesis may only be important for developing feeding structures precisely at the time of first feeding in more derived morphs with more specialised diets. These hypotheses, however, require further examination.

Environmental conditions experienced by the mother can often vary between different populations/morphs, even if populations/morphs share a migratory spawning strategy (i.e. temperature, water velocity, depth) that are known to have an effect on egg size (Rollinson & Hutchings, 2013; Jonsson & Jonsson, 2016). Mothers may be able to match the phenotype of their offspring to changes in the environment as a form of transgenerational plasticity (e.g. Agrawal et al., 1999; Storm & Lima, 2010; McGhee et al., 2012), such as adopting a bet-hedging strategy (Koops et al., 2003; Einum & Fleming, 2004; Marshall et al., 2008; Morrongiello et al., 2012). Size-correlated expression of genes may be a result of such maternal plasticity, whereby the mother can influence offspring development based on environmental cues (e.g. abundance of food: Hutchings, 1991) to improve fitness of offspring. Indeed, diet, stress and other environmental factors experienced by the mother can be transmitted across generation(s) via changes in DNA methylation/gene expression (Burdge et al., 2007; Franklin et al., 2010; Goerlich et al., 2012; Burton & Metcalfe, 2014), and has been attributed to the evolution of different morphs in response to the environment (Leimar et al., 2006). We suggest that the egg size-correlated expression of genes known to promote skeletogenesis (as seen here in VS at eye stage) suggests a maternally-mediated mechanism by which embryos can ossify relatively earlier or later to facilitate the transition to different environments that the offspring is likely to encounter. Relative gene expression patterns are reported for the anadomous population and four morphs of Arctic charr across four stages of early development, with evidence for egg-size correlated expression in all populations/morphs. The silver morph from lake Vatnshlíðarvatn shows the highest number of genes correlated with skeletogenesis, and also is the only morph to show egg size-correlated variation in craniofacial shape (Fig. 5.6; see below).

5.5.2 Craniofacial shape variation

We predicted that morphs that have a weaker magnitude of phenotypic divergence (VB and VS) should be more similar in shape than those more diverged sympatric morphs (TLB and TP). Contrary to this we found VB and VS to differ in craniofacial shape (before accounting for allometry) at hatching, with the brown morph having a widened hyoid arch in comparison to the silver morph, but this had disappeared at first feeding (Fig. 5.5). In the two more divergent sympatric morphs TLB and TP, no differences in craniofacial shape was seen at hatching, but craniofacial morphologies differed at first feeding both before and after accounting for allometry, with a wider hyoid arch and shorter Meckel’s cartilage in TLB compared to its sympatric TP morph. These results from TLB and TP are consistent with previous findings (Kapralova et al., 2015). Craniofacial shape differences between the two pairs of sympatric morphs may be adaptive and related to diet, although very little or no information are available on diet of first feeding fish of different morphs in the wild. Nevertheless, in the lab they were fed the same diet, yet differences between craniofacial shapes still occurred in both morphs, suggesting some degree of canalisation in craniofacial shape in the two þingvallavatn morphs. Juveniles and adults of these two morphs feed on very distinct prey in the wild, with the benthic morph (TLB) having a diet consisting of benthic invertebrates, whilst the pelagic planktivorous morph has a diet of planktonic crustaceans (Malmquist et al., 1992). Comparatively, the diet of both morphs in lake Vatnshlíðarvatn overlap considerably given the shallow nature of this lake, with both morphs feeding primarily on benthic organisms wherein seasonal variation in food abundance results in the silver morph becoming a more generalist feeder (Jónsson & Skúlason, 2000). The loss of craniofacial shape differences at first feeding (where both morphs were fed the same diet in the lab) likely reflects this overlap in diet, although differences in behaviour between the two morphs (both in the wild and in the lab) are unknown.

Previous studies suggest that offspring from different sized eggs diverge in feeding behaviour and mobility shortly after the onset of first feeding (Benhaïm et al., 2003; Leblanc et al., 2011). However, we found that evidence for egg size-correlated shape differences were weak (Table 5.4). Only the silver morph from lake Vatnshlíðarvatn showed any significant differences in craniofacial shape due to egg size, and this relationship was found at the first feeding stage (Table 5.4). Offspring from larger eggs had a more blunted snout, whilst offspring from smaller eggs are more contracted in shape at the terminal end of the Meckel's cartilage (Fig. 5.6). The influence of a maternally mediated trait (egg size) on trophic morphology at first feeding can potentially have an impact on what diet is available to her offspring, with egg size positively correlated to mouth gape (Knutsen & Tilseth, 1985), and ultimately promote morphological divergence. Interestingly, this morph also showed substantial egg size-correlated expression of genes related to skeletogenesis at eye stage (see above; Fig. 5.4), with larger eggs having higher relative expression (Fig. 5.4). At this stage of organogenesis, the formation of cartilage and skeletal elements provide the developmental roots for which any variants may stem from. As such, although egg size-correlated differences in gene expression at eye stage may be considered to be too early to influence trophic morphology, these developmental variants are likely to facilitate offspring shape variation by the onset of first feeding.

5.5.3 Covariance between gene expression and morphology along a gradient of sympatric divergence

Across all morphs used in this study, we found significant covariance between relative gene expression and craniofacial shape, with skeletal related genes primarily covarying with craniofacial shape at hatching, whilst at first feeding there was a switch to growth related genes being responsible for explaining much of the covariance between relative expression and craniofacial shape (Fig. 5.7). *Mmp9* (Matrix Metallopeptidase 9) had the highest loading on covariance with shape at hatching. This gene is involved in extra-cellular matrix (ECM) remodelling and plays an important role in physiological processes, including craniofacial development (Pedersen et al., 2015). Furthermore, *Mmp9* has also been identified as part of a conserved co-expression network involved in trophic divergence in Arctic charr (Ahi et al., 2014). Once hatched, the molecular processes underlying skeletogenesis likely facilitate the transition of offspring from endogeneous (yolk sac) to exogeneous feeding, since the ossification of trophic structures enable offspring to manipulate their food (Kapralova et al., 2015). Once feeding has started, *Star* (a gene for Steroidogenic Acute Regulatory protein) was found to have the highest loading on the covariance between craniofacial shape and relative expression of growth related genes, the covariance of which was correlated with egg size (Table 5.4). *Star* is associated with steroid and cortisol synthesis, and is thus involved in the stress response of fishes (Alsop & Vijayan, 2008). The relative expression levels of *Mmp9* and *Star* were also often correlated with egg size (see above). The size-correlations of *Star* at hatching, for example, showed alternative patterns in the sympatric morphs from lake Þingvallavatn, with the benthic morph having higher relative expression of *Star* in smaller eggs compared to its sympatric planktivorous morph, which has higher expression in larger eggs. Such patterns may be indicative towards the molecular mechanisms underlying the maintenance of alternative trophic polymorphisms in these sympatric morphs (Skúlason et al., 1989b).

We predicted that those morphs with a weaker magnitude of divergence would show more differences in shape and gene expression due to lack of canalisation. We provide support for this hypothesis as covariances between relative gene expression and craniofacial shape were more variable in the weakly diverged morphs from lake Vatnshlíðarvatn compared to other morphs, with VS being the primary driving force behind this significant covariance (Fig. 5.7; Table 5.5). Furthermore, VS was also the only morph to have craniofacial shape correlated with egg size, with larger eggs producing offspring with a phenotype that has a more bluntened snout in comparison to offspring from smaller eggs (Fig. 5.6). The silver morph not only drives much of the covariance between skeletal and growth related (relative) gene expression at hatching and first feeding (respectively; Fig. 5.7), but also has relatively higher expression of genes promoting skeletogenesis at eye stage (Fig. 5.3), that are correlated with egg size (Fig. 5.4), and even has egg size-correlated differences in shape (Fig. 5.6). The retention of such plasticity likely facilitates the switch between a generalist and a benthic diet in VS, depending upon seasonal variation in food abundance (Jónsson & Skúlason, 2000). Maternal effects may therefore act as a buffer in adverse environmental conditions by providing a source of plasticity for offspring, whilst genetic changes accumulate as the environment becomes more stable (Waddington, 1953). The VS morph likely experiences a lot of environmental heterogeneity based on the shallow nature of the lake, which can have numerous influences on environmental conditions with: 1) lack of water depth hindering the divergence of a truly benthic/pelagic morph; 2) the variation in temperature, especially in winter months where much of the lake freezes over; 3) the seasonal variation of food abundance; 4) its migratory life-history strategy; and 5) intraspecific competition with a more specialised brown morph (Jónsson & Skúlason, 2000). Remaining more plastic (likely mediated by maternal effects [egg size]) may facilitate the silver morph to readily respond to fluctuating environmental conditions. Collectively, our results demonstrate that maternal effects are able to influence the evolution and/or maintenance of alternative phenotypes, even in systems with very little resource availability.

5.5.4 Conclusion

We provide novel insight into how egg size-mediated maternal effects may play an important role in determining the developmental trajectories of offspring. To our knowledge, this is the first study examining the complex interplay between maternal effects (egg size), gene expression and craniofacial shape along a gradient of divergence in fishes. Maternal effects may be a driving mechanism underlying the rapid evolution of resource polymorphism, yet more studies are needed in fishes to determine to what extent maternal effects can have on evolutionary diversification.

5.6 References

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5.7 Appendix

Table S5.1 *A mixed effects model to test for the effects of developmental stage, morph (including the anadromous population), genes and their interaction on relative gene expression levels whilst accounting for family identity and cage as random effects nested within morph.*

	Random Effects				Fixed Effects					
♀	Variable	X2	X Df	*P*	Variable	Sum Sq	Num DF	Den DF	F	P
34	Family	0	1	1	**Dev.stage**	255	3	8967	248.64	**<.0001**
	Cage	152	1	<.0001	**Morph**	12	4	68	8.59	**<.0001**
					Genes	210	13	10255	47.18	**<.0001**
					Dev.stage*Morph	283	12	8688	69.05	**<.0001**
					Dev.stage*Genes	458	39	10255	34.36	**<.0001**
					Morph*Genes	386	52	10255	21.72	**<.0001**
					Dev.stage*Morph*Genes	1111	156	10255	20.84	**<.0001**

Dev.stage; developmental stage. Sum Sq; sum of squares. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Satterthwaite's approximations. F; F-value. *P; P*-value to determine the significance (bold) of these fixed effects on relative gene expression (log^2).

Table S5.2 *Mixed effect models for each gene per developmental stage to test for the effects of morph (including the anadromous population) on log^2 gene expression, whilst accounting for family identity and cage as random effects at: a) post-fertilisation, b) eye stage, c) hatching and d) first feeding. Cage was non-significant for all cases and therefore not shown here.*

a)

Gene identity (growth)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Star	183	0.00	1.000	2.4231	39.605	**<.0001**
Igf1	183	31.72	**<.0001**	0.54913	2.101	0.106
Gr	183	21.70	**<.0001**	1.6556	5.4509	**0.002**
Sgk1	183	0.03	0.900	0.55592	0.85614	0.503
Mtor	183	11.48	**0.001**	1.0071	2.255	0.087
Ghr1	182	2.87	0.090	3.9784	12.315	**<.0001**
Rictor	183	0.09	0.800	11.931	23.634	**<.0001**
Igf2	183	7.51	**0.006**	8.5008	5.0518	**0.003**

Gene identity (skeletal)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Timp2	183	10.60	**0.001**	0.96	1.35	0.277
Sparc	183	2.49	0.100	8.49	7.50	**0.0003**
Ctsk	183	8.10	**0.004**	2.36	4.73	**0.004**

Ets2	183	2.52	0.100	24.19	56.19	**<.0001**
Mmp2	182	10.10	**0.001**	2.02	1.92	0.134
Mmp9	182	14.44	**≤.0001**	4.92	1.68	0.181

b)

Gene identity (growth)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Star	184	6.27	**0.010**	7.54	21.11	**<.0001**
Igf1	184	20.00	**<.0001**	0.64	3.45	**0.020**
Gr	185	1.84	0.200	4.63	11.87	**<.0001**
Sgk1	185	6.41	**0.010**	3.65	13.38	**<.0001**
Mtor	183	10.30	**0.001**	1.84	5.97	**0.001**
Ghr1	184	0.10	0.800	3.65	12.01	**<.0001**
Rictor	184	9.42	**0.002**	0.84	4.44	**0.007**
Igf2	185	2.37	0.100	2.35	3.66	**0.015**

Gene identity (skeletal)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Timp2	34	0.00	1	339.14	32.586	**<.0001**
Sparc	34	0.00	1	101.71	46.116	**<.0001**
Ctsk	34	0.00	1	128.13	67.724	**<.0001**
Ets2	34	0.00	1	198.67	54.763	**<.0001**
Mmp2	34	0.00	1	145.51	53.798	**<.0001**
Mmp9	34	0.04	0.8	299.15	43.818	**<.0001**

c)

Gene identity (growth)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Star	34	19.20	**<.0001**	2.2874	3.3368	**0.023**
Igf1	34	0.73	0.400	0.23182	0.86095	0.499
Gr	34	4.95	**0.030**	7.9439	17.587	**<.0001**
Sgk1	34	12.10	**0.001**	4.1526	5.5193	**0.002**
Mtor	34	23.00	**<.0001**	1.6633	3.8753	**0.012**
Ghr1	34	4.10	**0.043**	0.91343	1.7466	0.166
Rictor	34	30.00	**<.0001**	1.0689	3.9652	**0.011**
Igf2	34	23.17	**<.0001**	0.9711	4.8226	**0.004**

Gene identity (skeletal)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Timp2	189	1.36	0.200	13.684	9.06	<**.0001**
Sparc	190	5.60	**0.020**	1.2228	2.09	0.107
Ctsk	189	0.08	0.800	6.5362	12.87	**<.0001**
Ets2	190	6.07	**0.010**	4.0084	6.03	**0.001**
Mmp2	189	14.10	**0.0002**	1.1491	2.08	0.108
Mmp9	190	8.93	**0.003**	4.7861	3.13	**0.029**

d)

Gene identity (growth)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Star	198	0	1.000	11.55	12.13	**<.0001**
Igf1	200	12.30	**0.0004**	2.26	7.53	**0.0003**
Gr	201	5.91	**0.020**	0.51	0.99	0.427
Sgk1	201	0.00	1.000	17.54	33.42	**<.0001**
Mtor	201	31.30	**<.0001**	2.37	3.42	**0.021**
Ghr1	201	35.77	**<.0001**	1.11	0.98	0.437
Rictor	200	58.00	**<.0001**	2.78	3.92	**0.011**
Igf2	201	22.00	**<.0001**	3.82	4.47	**0.006**
Gene identity (skeletal)	N	Morph:Family (Random Effect)		Morph (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Timp2	201	3.23	0.070	4.72	4.27	**0.007**
Sparc	201	24.67	**<.0001**	10.61	10.72	**<.0001**
Ctsk	201	30.30	**<.0001**	7.03	7.00	**0.0004**
Ets2	201	46.80	**<.0001**	3.18	3.35	**0.022**
Mmp2	201	28.70	**<.0001**	4.22	6.04	**0.001**
Mmp9	201	7.07	**0.008**	2.57	4.86	**0.004**

A total of 34 families per gene. Sum Sq; sum of squares. F; F-value. *P; P*-value to determine the significance (bold) of these fixed effects on relative gene expression (log^2).

Table S5.3 *Mixed effects models to determine whether gene expression differs for each gene across development whilst accounting for family identity and cage as random effects for all populations and morphs of Arctic charr: Fljótaá, FJ; Vatnshlíðarvatn silver, VS; þingvallavatn planktivorous, TP; þingvallavatn large benthic, TLB. Vatnshlíðarvatn brown (VB) results are not included, but see Beck et al., (2018). Only those significant random effects are shown for each population and morph.*

Fljotáa (FJ)						
Gene identity (growth)	N	Family (Random Effect)		Developmental stage (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Star	128	0.00	1	35.708	65.426	**0.000**
Igf1	128	0.00	1	128	58.848	**0.000**
Gr	128	0.00	1	118.01	12.008	**0.000**
Sgk1	128	0.00	1	124	7.0107	**0.000**
Mtor	128	0.00	1	124	6.3965	**0.000**
Ghr1	128	0.00	1	124	4.0653	**0.009**
Rictor	127	0.00	1	123	19.451	**0.000**
Igf2	128	0.54	0.5	119.57	24.992	**0.000**
Gene identity (skeletal)	N	X^2	*P*	Sum Sq	F	*P*

Timp2	128	6.24	**0.01**	119.19	21.058	**0.000**
Sparc	128	0.00	1	124	21.454	**0.000**
Ctsk	128	0.00	1	124	30.054	**0.000**
Ets2	128	0.00	1	117.72	43.545	**0.000**
Mmp2	128	0.00	1	120.5	35.434	**0.000**
Mmp9	128	0.00	1	124	6.3017	**0.001**

Vatnshlíðarvatn silver (VS)				
Gene identity (growth)	N	Developmental stage (Fixed Effect)		
		Sum Sq	F	*P*
Star	115	36.73	41.23	**0.000**
Igf1	117	21.95	100.38	**0.000**
Gr	118	27.76	37.96	**0.000**
Sgk1	118	2.60	3.55	**0.017**
Mtor	118	3.74	5.45	**0.002**
Ghr1	117	9.24	13.52	**0.000**
Rictor	118	2.16	6.12	**0.001**
Igf2	118	6.44	5.76	**0.001**
Gene identity (skeletal)	N	Sum Sq	F	*P*
Timp2	118	258.97	19.55	**0.000**
Sparc	119	94.53	40.06	**0.000**
Ctsk	117	151.90	60.71	**0.000**
Ets2	119	263.10	63.99	**0.000**
Mmp2	118	124.40	41.63	**0.000**
Mmp9	118	326.33	37.86	**0.000**

Þingvallavatn planktivorous (TP)						
Gene identity (growth)	N	Family (Random Effect)		Developmental stage (Fixed Effect)		
		X^2	*P*	Sum Sq	F	*P*
Star	135	5.78	**0.020**	21.67	95.36	**0.000**
Igf1	137	8.63	**0.003**	8.82	70.67	**0.000**
Gr	137	2.49	0.100	0.30	7.44	**0.000**
Sgk1	137	8.55	**0.003**	2.57	23.47	**0.000**
Mtor	137	5.51	**0.020**	1.23	40.94	**0.000**
Ghr1	137	0.82	4.000	1.83	31.54	**0.000**
Rictor	137	4.18	**0.040**	1.38	41.16	**0.000**
Igf2	137	2.99	**0.080**	9.08	199.41	**0.000**
Gene identity (skeletal)	N	X^2	*P*	Sum Sq	F	*P*
Timp2	137	13.50	**0.000**	9.08	79.65	**0.000**

Sparc	137	10.90	**0.001**	1.67	37.03	**0.000**
Ctsk	137	1.11	0.300	0.84	8.75	**0.000**
Ets2	137	6.52	**0.010**	11.19	22.04	**0.000**
Mmp2	136	7.64	**0.006**	5.98	18.46	**0.000**
Mmp9	137	2.42	0.100	4.03	18.45	**0.000**

þingvallavatn large benthic (TLB)				
Gene identity (growth)	N	Developmental stage (Fixed Effect)		
		Sum Sq	F	*P*
Star	104	20.50	81.60	**0.000**
Igf1	105	20.43	196.54	**0.000**
Gr	105	3.78	20.94	**0.000**
Sgk1	105	1.72	8.88	**0.000**
Mtor	105	10.41	40.14	**0.000**
Ghr1	105	0.55	5.10	**0.003**
Rictor	105	4.39	23.75	**0.000**
Igf2	105	7.03	17.67	**0.000**
Gene identity (skeletal)	N	Sum Sq	F	*P*
Timp2	105	2.33	16.48	**0.000**
Sparc	105	6.83	25.44	**0.000**
Ctsk	105	0.87	3.96	**0.010**
Ets2	105	36.23	238.64	**0.000**
Mmp2	105	2.17	5.71	**0.001**
Mmp9	105	33.26	52.71	**0.000**

Table S5.4 *A mixed effects model for each population and morph (FJ, Fljotaa; VB, Vatnshlíðarvatn brown; VS, Vatnshlíðarvatn silver; TLB, þingvallavatn large benthic; TP, þingvallavatn planktivorous) of Arctic charr to test for the effects of developmental stage, gene identity and their interaction whilst accounting for family identity and cage as random effects.*

Morph	♀ N	N	Response	Random Effects				Fixed Effects					
				Variable	X^2	Chi. DF	*P*	Variable	Sum Sq	Num DF	Den DF	F	*P*
FJ	6	1791	Log expression	Family	0.00	1	1	**Genes**	38.74	13	1727	17.34	<0.0001
				Cage	1.04	1	0.3	**Dev.stage**	66.02	3	959	128.07	<0.0001
								Genes*Dev.stage	85.47	39	1727	12.75	<0.0001
VB	7	2164		**Family**	13.48	1	0.0002	**Genes**	70.74	13	2094	16.94	<0.0001
				Cage	0.20	1	0.7	**Dev.stage**	51.38	3	1900	53.33	<0.0001
								Genes*Dev.stage	175.72	39	2094	14.03	<0.0001
VS	5	1648		Family	0.00	1	1	**Genes**	305.02	13	1587	25.27	<0.0001
				Cage	10.40	1	0.001	**Dev.stage**	344.67	3	1588	123.76	<0.0001
								Genes*Dev.stage	969.21	39	1587	26.77	<0.0001
TLB	5	1469		Family	3.59	1	0.06	**Genes**	69.57	13	1409	62.59	<0.0001
				Cage	0.00	1	1	**Dev.stage**	2.59	3	1407	10.09	<0.0001
								Genes*Dev.stage	148.21	39	1409	44.45	<0.0001
TP	6	1915		**Family**	4.63	1	0.03	**Genes**	24.96	13	1854	37.37	<0.0001
								Dev.stage	11.21	3	1855	72.73	<0.0001
								Genes*Dev.stage	68.99	39	1854	34.43	<0.0001

Dev.stage; developmental stage. Sum Sq; sum of squares. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Satterthwaite's approximations. F; F-value. *P; P*-value to determine the significance of these fixed effects on relative gene expression ($\log^2$). Note that TP does not have cage as a random effect since there was only one cage per family for this morph.

Table S5.5 *Model selection results using the Akaike Information Criterion (AIC) to determine whether gene identity (fixed factor), individual size (covariate), as well as their interaction on gene expression was best fitted using a linear or non-linear (polynomial) model across four developmental stages (PF, post-fertilisation; E, eye stage; H, hatching; and FF, first feeding) and four populations/morphs of Arctic charr: a) Fljotaa, FJ; b) Vatnshlíðarvatn silver, VS; c) þingvallavatn large benthic, TLB; and d) þingvallavatn planktivorous, TP (for VB see Beck et al., 2018, Paper II). The model included family identity and cage as random effects for all morphs except TP (family only) to account for non-independence of data.*

a)

Fljotáa (FJ)						
Dev. stage	**Model**	**Df**	**AIC**	X^2	**Df**	***P***
PF	**Linear**	31	564			
	Polynomial^2	45	581	11.21	14	0.669
	Polynomial^3	59	600	8.61	14	0.855
E	**Linear**	31	366			
	Polynomial^2	45	384	9.21	14	0.817
	Polynomial^3	59	404	8.74	14	0.847
H	**Linear**	31	-122			
	Polynomial^2	45	-111	16.41	14	0.289
	Polynomial^3	59	-86	3.42	14	0.998
FF	**Linear**	31	743			
	Polynomial^2	45	768	3.39	14	0.998
	Polynomial^3	59	779	17.11	14	0.250

b)

Vatnshlíðarvatn silver (VS)						
Dev. stage	**Model**	**Df**	**AIC**	X^2	**Df**	***P***
PF	Linear	31	417			
	Polynomial^2	45	415	29.94	14	0.008
	Polynomial^3	59	407	36.18	14	0.001
E	**Linear**	31	1499			
	Polynomial^2	45	1520	6.84	14	0.941
	Polynomial^3	59	1538	9.91	14	0.769
H	Linear	31	962			
	Polynomial^2	45	944	46.53	14	0.000
	Polynomial^3	59	951	20.54	14	0.114
FF	**Linear**	31	710			
	Polynomial^2	45	721	16.87	14	0.263
	Polynomial^3	59	713	36.11	14	0.001

c)

þingvallavatn large benthic (TLB)						
Dev. stage	**Model**	**Df**	**AIC**	X^2	**Df**	***P***
PF	Linear	31	441			
	Polynomial^2	45	414	54.84	14	0.000
	Polynomial^3	59	386	55.89	14	0.000
E	**Linear**	31	5			
	Polynomial^2	45	20	13.04	14	0.523
	Polynomial^3	59	33	14.10	14	0.443
H	**Linear**	31	-198			
	Polynomial^2	45	-188	18.32	14	0.193
	Polynomial^3	59	-181	20.90	14	0.104
FF	**Linear**	31	-79			
	Polynomial^2	45	-55	4.09	14	0.995
	Polynomial^3	59	-38	10.76	14	0.705

d)

þingvallavatn planktivorous (TP)						
Dev. stage	**Model**	**Df**	**AIC**	X^2	**Df**	***P***
PF	Linear	30	389			
	Polynomial^2	44	365	51.97	14	0.000
	Polynomial^3	58	384	9.33	14	0.809
E	**Linear**	30	-466			
	Polynomial^2	44	-460	21.50	14	0.090
	Polynomial^3	58	-446	14.64	14	0.403
H	**Linear**	30	-431			
	Polynomial^2	44	-421	17.75	14	0.218
	Polynomial^3	58	-410	17.20	14	0.246
FF	**Linear**	30	-499			
	Polynomial^2	44	-503	31.83	14	0.004
	Polynomial^3	58	-499	24.29	14	0.042

Dev.stage, developmental stage. Df, degrees of freedom. AIC, Akaike Information Criterion. X^2, chi square.

Table S5.6 *Model selection results using the Akaike Information Criterion (AIC) to determine whether the expression of individual genes was best fitted using a linear or non-linear (polynomial) model across four developmental stages (PF, post-fertilisation; E, eye stage; H, hatching; and FF, first feeding) in the anadromous population and three morphs of Arctic charr: a) Fljotaa, FJ; b) Vatnshlíðarvatn silver, VS; c) þingvallavatn large benthic, TLB; and d) þingvallavatn planktivorous, TP (for VB see Paper II). The model included family identity and cage as random effects for all morphs except TP (family only) to account for non-independence of data.*

a)

Fljótaá (FJ)				
Gene	**PF**	**E**	**H**	**FF**
Star	-	Linear	Polynomial^2	Linear
Igf1	Linear	Linear	Linear	Polynomial^3
Gr	Polynomial^2	Linear	Linear	Linear
Sgk1	Linear	Linear	Linear	Linear
Mtor	Linear	Linear	Linear	Linear
Ghr1	Linear	Linear	Linear	Linear
Rictor	Linear	Linear	Linear	Linear
Igf2	Linear	Linear	Linear	Linear
Timp2	Linear	Linear	Linear	Linear
Sparc	Linear	Linear	Linear	Linear
Ctsk	Linear	Linear	Linear	Linear
Ets2	Polynomial^3	Linear	Linear	Linear
Mmp2	Polynomial^2	Linear	Polynomial^2	Linear
Mmp9	Linear	Linear	Linear	Polynomial^3

b)

Vatnshlíðarvatn silver (VS)				
Gene	**PF**	**E**	**H**	**FF**
Star	-	Polynomial^3	Linear	Polynomial^3
Igf1	-	Polynomial^3	Polynomial^2	Polynomial^2
Gr	Linear	Linear	Linear	Linear
Sgk1	Linear	Linear	Polynomial^3	Polynomial^3
Mtor	Linear	Linear	Polynomial^2	Linear
Ghr1	Linear	Linear	Linear	Linear
Rictor	Linear	Linear	Linear	Polynomial^2
Igf2	Linear	Linear	Polynomial^3	Linear
Timp2	Linear	Linear	Polynomial^2	Polynomial^3
Sparc	Polynomial^2	Linear	Polynomial^3	Linear
Ctsk	Linear	Linear	Polynomial^3	Linear
Ets2	-	Linear	Linear	Linear
Mmp2	Polynomial^2	Linear	Linear	Linear
Mmp9	Linear	Linear	Linear	Linear

c)

þingvallavatn large benthic (TLB)				
Gene	**PF**	**E**	**H**	**FF**
Star	-	Linear	Linear	Linear
Igf1	-	Linear	Linear	Linear
Gr	Linear	Linear	Linear	Linear
Sgk1	Polynomial^{3}	Polynomial^{3}	Linear	Linear
Mtor	Polynomial^{3}	Linear	Linear	Linear
Ghr1	Polynomial^{3}	Linear	Polynomial^{3}	Polynomial^{3}
Rictor	Polynomial^{3}	Linear	Polynomial^{3}	Linear
Igf2	Polynomial^{2}	Linear	Linear	Linear
Timp2	Polynomial^{2}	Linear	Polynomial^{2}	Linear
Sparc	Linear	Linear	Linear	Linear
Ctsk	Polynomial^{3}	Linear	Linear	Linear
Ets2	-	Linear	Polynomial^{2}	Linear
Mmp2	Polynomial^{3}	Linear	Linear	Polynomial^{2}
Mmp9	Polynomial^{2}	Polynomial^{2}	Polynomial^{2}	Linear

d)

þingvallavatn planktivorous (TP)				
Gene	**PF**	**E**	**H**	**FF**
Star	Linear	Linear	Linear	Linear
Igf1	Linear	Polynomial^{2}	Linear	Polynomial^{2}
Gr	Linear	Polynomial^{2}	Linear	Polynomial^{3}
Sgk1	Polynomial^{3}	Linear	Polynomial^{2}	Linear
Mtor	Polynomial^{3}	Linear	Polynomial^{3}	Polynomial^{3}
Ghr1	Linear	Polynomial^{3}	Linear	Polynomial^{2}
Rictor	Polynomial^{2}	Linear	Linear	Polynomial^{3}
Igf2	Polynomial^{3}	Linear	Linear	Polynomial^{3}
Timp2	Linear	Polynomial^{3}	Linear	Polynomial^{2}
Sparc	Linear	Polynomial^{3}	Polynomial^{3}	Polynomial^{3}
Ctsk	Linear	Polynomial^{2}	Linear	Linear
Ets2	Polynomial^{2}	Linear	Linear	Polynomial^{3}
Mmp2	Polynomial^{2}	Linear	Linear	Polynomial^{3}
Mmp9	Linear	Linear	Polynomial^{2}	Polynomial^{3}

Table S5.7 *Results of mixed effects models based on best-fitted models per gene (see Table S5.6 testing for the effect of gene identity (fixed factor) and individual size (covariate), as well as their interaction, on gene expression in three Arctic charr morphs: a) Vatnshlíðarvatn silver (VS), b) þingvallavatn large benthic (TLB) and c) þingvallavatn planktivorous (TP). Gene specific model fits for the anadromous Fljótaá did not show any different results and are therefore not shown. These analyses were conducted separately for each developmental stage due to significant differences in gene expression among developmental stages (see Table S5.1). Significance of random (family and cage) and fixed effects is included for the overall models. Only those developmental stages that were significant are shown.*

a)

Vatnshlíðarvatn silver (VS)																		
Dev. stage	N	N fam.	Response	Random Effects				Fixed Effects										
				Variable	X^2	*X* Df	*P*	Variable	Sum Sq	Num DF	Den DF	F	*P*	β	SE	Df	*t*	*P*
PF	418	5	Log expression	**Family**	41.2	1	**<.0001**	**Size**	1.60	1	133	11.32	0.001					
								Genes	5.23	13	386	2.84	0.001					
								Size*Genes	7.08	13	386	3.84	<.0001					
								Igf2						0.98	0.28	374	3.47	**0.008**
								Mmp9						-1.05	0.28	374	-3.70	**0.004**

b)

þingvallavatn large benthic (TLB)																		
Dev. stage	N	N fam.	Response	Random Effects				Fixed Effects										
				Variable	X^2	*X* Df	P	Variable	Sum Sq	Num DF	Den DF	F	*P*	β	SE	Df	*t*	*P*
PF	378	5	Log expression	**Family**	8.98	1	0.003	Size (poly^2)	4.77	2	279	15.84	**<.0001**					
				Cage	0.00	1	1	Genes	176.70	13	332	90.35	**<.0001**					
								Size*Genes	12.86	26	332	3.29	**<.0001**					
								Igf2						1.86	0.39	335	4.81	**<.0001**

H	294	5		Family	0.15	1	0.7	Size (poly^3)	0.07	3	19	0.95	0.436					
				Cage	17.04	1	<.0001	**Genes**	12.15	13	228	39.03	**<.0001**					
								Size*Genes	2.13	39	228	2.28	**0.0001**					
								Ghr1						0.31	0.09	234	3.45	**0.009**

c)

þingvallavatn planktivorous (TP)																		
Dev. stage	N	N fam.	Response	Random Effects				Fixed Effects										
				Variable	X^2	*X* Df	P	Variable	Sum Sq	Num DF	Den DF	F	*P*	β	SE	Df	*t*	*P*
H	504	6	Log expression	**Family**	86	1	**<.0001**	Size (poly^2)	0.09	2	59	1.95	0.152					
								Genes	20.55	13	457	69.5	<.0001					
								Size*Genes	1.24	26	457	2.10	0.001					
								Sgk1						0.09	0.03	350	2.92	0.050
FF	502	6		**Family**	17.5	1	**<.0001**	**Size**	0.20	1	32	10.06	0.003					
								Genes	0.49	13	468	1.91	0.027					
								Size*Genes	0.73	13	468	2.82	0.001					
								Star						0.10	0.02	317	5.34	<.0001
								Sgk1						0.06	0.02	318	2.99	0.041
FF	502	6		**Family**	134	1	**<.0001**	**Size (poly^3)**	0.58	3	81	10.38	<.0001					
								Genes	9.13	13	440	37.41	<.0001					
								Size*Genes	1.34	39	440	1.84	0.002					
								Mmp9						0.11	0.03	438	3.28	0.015

Dev.stage; developmental stage. PF; post-fertilisation. E; Eye stage. H; hatching. FF; first feeding. N; total number of observations across all individuals and genes. Log expression; log2 (relative mRNA expression+1). X^2; Chi square statistic. Chi. DF; number of degrees of freedom for the test. *P*; *P*-value of the likelihood ratio test for the random effect. Sum Sq; sum of squares. N fam.; number of families. Num DF; numerator degrees of freedom. Den DF; denominator degrees of freedom based on Satterthwaite's approximations. F; F-value. Gene slopes that remained significantly different from zero after Tukey's adjustments are indicated, as well as slope (β), standard error (SE), degrees of freedom (DF), t-ratio (*t*) and associated *P*-values. Significant variables in bold. PF; post-fertlisation. E; eye stage. H; hatching stage. FF; first feeding stage.

Table S5.8 *Pairwise comparisons of slopes based on the significant interaction between size x genes on relative gene expression in the anadromous population and four morphs of Arctic charr: FJ, Fljótaá; VB, Vatnshlíðarvatn brown; VS, Vatnshlíðarvatn silver; TLB, Þingvallavatn large benthic; and TP, Þingvallavatn planktivorous, across four developmental stages, a) post-fertilisation, b) eye stage, c) hatching, and d) first feeding.*

a) Post-fertilisation

Morph	Contrast		Estimate	SE	df	*t*	*P*
FJ	Ctsk	Mmp9	-1.50	0.40	312	-3.72	0.017
	Ghr1	Mmp9	-1.53	0.40	312	-3.80	0.013
	Mmp9	Rictor	1.40	0.40	312	3.48	0.037
VB	Ctsk	Mmp9	-0.94	0.18	503	-5.18	<.0001
	Ets2	Mmp9	-1.04	0.18	503	-5.72	<.0001
	Ghr1	Mmp9	-1.07	0.18	503	-5.92	<.0001
	Gr	Mmp9	-1.19	0.18	503	-6.58	<.0001
	Igf1	Mmp9	-1.10	0.18	503	-6.05	<.0001
	Igf2	Mmp9	-0.87	0.18	503	-4.81	0.0002
	Mmp2	Mmp9	-0.96	0.18	503	-5.29	<.0001
	Mmp9	Mtor	0.92	0.18	503	5.09	<.0001
	Mmp9	Rictor	1.07	0.18	503	5.93	<.0001
	Mmp9	Sgk1	0.83	0.18	503	4.60	0.0004
	Mmp9	Sparc	1.23	0.18	503	6.82	<.0001
	Mmp9	Star	1.04	0.18	503	5.72	<.0001
	Mmp9	timp2	0.96	0.18	503	5.28	<.0001
VS	Ghr1	Mmp9	2.19	0.60	358	3.64	0.022
	Rictor	Mmp9	2.19	0.60	358	3.67	0.019
	Igf2	Mmp9	2.05	0.60	358	3.45	0.0398
	Timp2	Mmp9	2.58	0.60	358	4.34	0.002
	Sparc	Mmp9	3.36	0.60	358	5.65	<.0001
	Mmp2	Mmp9	2.37	0.60	358	3.98	0.006
TLB	Ctsk	Igf2	-3.90	0.92	313	-4.25	0.002
	Ctsk	Mmp2	-3.17	0.92	313	-3.45	0.041
TP	Star	Ets2	1.71	0.30	400	5.75	<.0001
	Igf1	Ets2	1.88	0.30	400	6.34	<.0001
	Gr	Ets2	1.82	0.30	400	6.11	<.0001
	Sgk1	Ets2	1.89	0.30	400	6.37	<.0001
	Mtor	Ets2	1.73	0.30	400	5.83	<.0001
	Ghr1	Ets2	1.57	0.30	400	5.30	<.0001
	Rictor	Ets2	1.54	0.30	400	5.20	<.0001
	Igf2	Ets2	1.39	0.30	400	4.66	0.0004
	Timp2	Ets2	2.03	0.30	400	6.83	<.0001
	Sparc	Ets2	1.73	0.30	400	5.83	<.0001

	Ctsk	Ets2	1.95	0.30	400	6.56	<.0001
	Ets2	Mmp2	-1.10	0.30	400	-3.69	0.018
	Ets2	Mmp9	-1.63	0.30	400	-5.48	<.0001

b) Eye stage

Morph	Contrast		Estimate	SE	df	*t*	*P*
VB	Ets2	Mmp2	0.44	0.12	445	3.65	0.020
	Ets2	Mmp9	0.42	0.12	445	3.48	0.036
	Igf2	Mmp2	0.42	0.12	445	3.52	0.032
	Mmp2	Mtor	-0.45	0.12	445	-3.72	0.016
	Mmp2	Rictor	-0.48	0.12	445	-3.96	0.007
	Mmp2	Sgk1	-0.50	0.12	445	-4.14	0.003
	Mmp9	Mtor	-0.43	0.12	445	-3.55	0.028
	Mmp9	Rictor	-0.45	0.12	445	-3.79	0.013
	Mmp9	Sgk1	-0.48	0.12	445	-3.97	0.006
VS	Sgk1	Igf2	-0.42	0.11	385	-3.78	0.013
TP	Star	Timp2	0.38	0.10	429	3.75	0.015
	Star	Mmp9	0.42	0.10	429	4.19	0.003
	Igf1	Mmp9	0.35	0.10	429	3.49	0.034
	Sgk1	Mmp9	0.36	0.10	429	3.59	0.025
	Ghr1	Mmp9	0.37	0.10	429	3.68	0.018

c) Hatching

Morph	Contrast		Estimate	SE	df	*t*	*P*
FJ	Ctsk	Mmp9	-0.37	0.06	450	-6.00	<.0001
	Ets2	Mmp9	-0.25	0.06	450	-4.15	0.003
	Ghr1	Mmp9	-0.24	0.06	450	-3.96	0.007
	Gr	Mmp9	-0.32	0.06	450	-5.23	<.0001
	Igf1	Mmp9	-0.35	0.06	450	-5.66	<.0001
	Igf2	Mmp9	-0.30	0.06	450	-4.93	0.0001
	Mmp2	Mmp9	-0.35	0.06	450	-5.64	<.0001
	Mmp9	Mtor	0.29	0.06	450	4.79	0.0002
	Mmp9	Rictor	0.30	0.06	450	4.82	0.0002
	Mmp9	Sgk1	0.31	0.06	450	5.02	0.0001
	Mmp9	Sparc	0.33	0.06	450	5.30	<.0001
	Mmp9	Star	0.23	0.06	450	3.72	0.016
	Mmp9	Timp2	0.32	0.06	450	5.15	<.0001
VB	Ctsk	Sgk1	-0.40	0.08	515	-5.01	0.0001
	Ets2	Sgk1	-0.38	0.08	515	-4.79	0.0002
	Ghr1	Sgk1	-0.46	0.08	515	-5.83	<.0001
	Gr	Sgk1	-0.33	0.08	515	-4.16	0.003

	Igf1	Sgk1	-0.30	0.08	515	-3.74	0.015
	Igf2	Sgk1	-0.49	0.08	515	-6.21	<.0001
	Mmp2	Sgk1	-0.48	0.08	515	-6.13	<.0001
	Mmp9	Sgk1	-0.52	0.08	515	-6.63	<.0001
	Mmp9	Star	-0.30	0.08	515	-3.77	0.013
	Mtor	Sgk1	-0.51	0.08	515	-6.46	<.0001
	Mtor	Star	-0.28	0.08	515	-3.60	0.024
	Rictor	Sgk1	-0.48	0.08	515	-6.01	<.0001
	Sgk1	Sparc	0.42	0.08	515	5.30	<.0001
	Sgk1	Timp2	0.33	0.08	515	4.20	0.003
TLB	Ctsk	Star	0.21	0.06	256	3.52	0.033
	Ets2	Ghr1	-0.20	0.06	256	-3.38	0.051
	Ghr1	Sgk1	0.22	0.06	256	3.77	0.014
	Ghr1	Star	0.31	0.06	256	5.30	<.0001
	Ghr1	Timp2	0.25	0.06	256	4.21	0.003
	Igf1	Star	0.24	0.06	256	4.12	0.004
	Igf2	Star	0.20	0.06	256	3.39	0.050
	Mmp2	Star	0.26	0.06	256	4.39	0.001
	Sparc	Star	0.24	0.06	256	4.15	0.004
TP	Star	Ets2	0.15	0.04	471	3.87	0.009
	Sgk1	Ets2	0.13	0.04	471	3.35	0.054
	Mtor	Timp2	-0.13	0.04	471	-3.38	0.049
	Rictor	Timp2	-0.14	0.04	471	-3.46	0.038
	Timp2	Ets2	0.17	0.04	471	4.35	0.001

d) First feeding

Morph	Contrast		Estimate	SE	df	*t*	*P*
VB	Ghr1	Gr	-0.30	0.08	532	-3.82	0.011
	Ghr1	Timp2	-0.28	0.08	532	-3.53	0.030
VS	Igf2	Sgk1	0.83	0.22	357	3.78	0.013
TP	Star	Gr	0.09	0.03	455	3.58	0.026
	Star	Igf2	0.10	0.03	455	4.04	0.005
	Star	Sparc	0.11	0.03	455	4.32	0.002
	Star	Ctsk	0.09	0.03	455	3.56	0.027
	Star	Ets2	0.12	0.03	455	4.77	0.0002
	Star	Mmp2	0.09	0.03	455	3.74	0.015
	Ghr1	Ets2	0.08	0.02	455	3.37	0.05

***Table S5.9** Procrustes ANOVA examining the effect of morph on partial least square (PLS) components explaining the most amount of covariance (PLS1) between craniofacial shape in the anadromous population and three morphs of Arctic charr (FJ, Fljótaá, VB, Vatnshlíðarvatn brown, VS, Vatnshlíðarvatn silver and TP, Þingvallavatn planktivorous) and relative expression levels of: a) growth-, and b) skeletal- related genes at hatching, c) growth- and d) skeletal- related genes at first feeding. Note that for brevity, the anadromous population is included within 'morph'.*

a) Growth related genes at hatching
(PLS1 = 73% covariance)

	Df	SS	R^2	F	Z	*P*
Morph	3	0.01	0.36	3.68	2.20	**0.032**
Residuals	20	0.02				

b) Skeletal related genes at hatching
(PLS1 = 65% covariance)

	Df	SS	R^2	F	Z	*P*
Morph	3	0.00	0.55	8.21	3.39	**0.001**
Residuals	20	0.00				

c) Growth related genes at first feeding
(PLS1 = 91% covariance)

	Df	SS	R^2	F	Z	*P*
Morph	3	0.01	0.48	6.09	2.99	**0.004**
Residuals	20	0.01				

d) Skeletal related genes at first feeding
(PLS1 = 89% covariance)

	Df	SS	R^2	F	Z	*P*
Morph	3	0.01	0.47	5.99	2.96	**0.004**
Residuals	20	0.01				

***Table S5.10** Procrustes ANOVA examining the effect of morph (including the anadromous population), egg size, and their interaction on partial least square (PLS) components explaining the most amount of covariance (PLS1) between craniofacial shape and relative expression levels of growth related genes at first feeding in an anadromous population and three morphs of Arctic charr (FJ, Fljótaá, VB, Vatnshlíðarvatn brown, VS, Vatnshlíðarvatn silver and TP, Þingvallavatn planktivorous). Effect sizes (Z) in the bottom half of matrices and associated P values above.*

	FJ	TP	VB	VS
FJ	-	0.499	0.837	0.498
TP	0.853	-	0.510	0.500
VB	0.209	0.816	-	0.499
VS	0.946	0.973	0.956	-