UNIVERSITY OF ICELAND

Ph.D. Dissertation

in Biology

Phenotypic plasticity in Mývatn threespine stickleback (*Gasterosteus aculeatus*): response to temperature and diet within and across generations

Alessandra Schnider

February 2025

FACULTY OF LIFE AND ENVIRONMENTAL SCIENCES

Phenotypic plasticity in Mývatn threespine stickleback (*Gasterosteus aculeatus*): response to temperature and diet within and across generations

Alessandra Schnider

Dissertation submitted in partial fulfilment of a
Philosophiae Doctor degree in Biology

Ph.D. Committee
Bjarni Kristófer Kristjánsson
Katja Räsänen
Zophonías Oddur Jónsson
Blake Matthews
Catherine Peichel

Opponents
Lisa N. S. Shama
Antti P. Eloranta

Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
University of Iceland
Reykjavik, February 2025

Phenotypic plasticity in Mývatn threespine stickleback (*Gasterosteus aculeatus*): response to temperature and diet within and across generations

Dissertation submitted in partial fulfilment of a *Ph.D* in Biology

Faculty of Life and Environmental Sciences
School of Engineering and Natural Sciences
University of Iceland
Sturlugötu 7
102, Reykjavík
Iceland

Telephone: 525 4000

Bibliographic information:
Alessandra Schnider, 2025, *Phenotypic plasticity in Mývatn threespine stickleback (Gasterosteus aculeatus): response to temperature and diet within and across generations*, Ph.D. dissertation, Faculty of Life and Environmental Sciences, University of Iceland, 111 pp.

Author ORCID: 0009-0007-2634-8493
ISBN: 978-9935-9807-9-3

Abstract

Phenotypic plasticity, the ability of a single genotype to produce different phenotypes in response to environmental stimuli, can mitigate effects of adverse environmental conditions within and across generations and be an important source of adaptation in response to natural selection. The aim of this thesis was to study how environmental drivers shape the phenotype of threespine stickleback, within and across generations, in the spatio-temporally varying system, Lake Mývatn, Iceland. Firstly, my study of diet and morphology of wild juvenile Mývatn stickleback revealed a dietary niche shift from Cladocera to midge larvae with increasing body size and diverging head morphology in response to varying diet composition. Secondly, I conducted a multigenerational plasticity experiment, rearing offspring from stickleback originating from two contrasting habitats under varying temperature and diet conditions. I analysed phenotypic information of juveniles and sexually mature females and males across two generations. I further collected liver samples of sexually mature experimental and wild individuals for transcriptomic analyses, to further our understanding of the underlying molecular mechanisms. I found clear environmental effects on traits directly associated with fitness. Thus, transgenerational effects appear crucial in shaping biological fitness in this highly dynamic system. I also identified differentially expressed genes in response to temperature and diet, potentially reducing environmental stress and support habitat expansion. This thesis enhances our understanding of phenotypic plasticity and transgenerational effects in dynamic systems. Integrating multiple environmental factors over biologically relevant timeframes is rare, making my findings crucial for understanding how phenotypic plasticity contributes to biological diversity.

Útdráttur

Sveigjanlegt svipfar, geta arfgerðar til að mynda mismunandi svipgerðir sem svar við breytilegu umhverfi, getur dregið úr áhrifum erfiðra umhverfisaðstæðna, innan og milli kynslóða, og er mikilvægt í aðlögunarsvörun vegna náttúrulegs vals. Helsta markmið verkefnisins var að rannsaka mótandi áhrif umhverfisþátta á svipgerð hornsíla, innan og milli kynslóða, í tíma og rúmi. Ég rannsakaði fæðu og fæðutengt útlit hornsílaseiða í hinu síbreytilega Mývatni. Þegar að seiðin lengdust breyttist fæða þeirra frá vatnaflóm til mýlirfa og höfuðlögun tengdist fæðunni. Ég mældi sveigjanlegt svipfar innan og milli kynslóða í tilraun þar sem að hornsíli frá tveimur svæðum voru alin við ólíkt hitastig og fæðu í tvær kynslóðir og upplýsingum um svipgerð seiða og kynþroska hænga og hrygna var safnað. Sýni til mælinga á genatjáningu voru tekin af lifur kynþroska einstaklinga úr Mývatni og afkvæma úr tilrauninni, til að skilja betur þá erfðafræðilegu ferla sem móta svipgerðarfjölbreytileikann sem sást. Umhverfið hafði greinileg áhrif á þætti sem tengja má beint við hæfni. Þættir sem berast milli kynslóða eru greinilega mikilvægir fyrir líffræðilega hæfni í þessu kvika kerfi. Nokkur gen fundust sem sýndu mismunandi tjáningu í tengslum við hitastig og fæðu. Þau geta mögulega dregið úr streituáhrifum tengdum umhverfi og gert einstaklingum kleift að nema ný búsvæði. Niðurstöðurnar auka skilning á mikilvægi sveigjanlegs svipfars, innan og milli kynslóða, fyrir einstaklinga í kviku vistkerfi. Rannsóknir þar sem skoðaðir eru margir umhverfisþættir á líffræðilega mikilvægum tímaskala eru fáar. Því eru niðurstöður mínar sérlega mikilvægar fyrir skilning á hvernig sveigjanlegt svipfar hefur áhrif á líffræðilega fjölbreytni.

Dedication

To Dr. Barbara Glutz-Maier. The first woman in my life with a PhD,

and who inspired, encourage, and supported me

throughout my academic studies.

Table of Contents

List of Figures

List of Tables

List of Papers

This thesis is based on the following three scientific papers, which are referred to in the text by their Roman numerals (I, II, III):

I. Juvenile feeding ecology of threespine stickleback (*Gasterosteus aculeatus*): Alessandra Schnider, Franka Hemme, Etienne de la Burgade, Bjarni Kristófer Kristjánsson; Paper submitted to Aquatic Ecology: Special Issue on Mývatn Research.

II. Plasticity in key life history traits of threespine stickleback (*Gasterosteus aculeatus*) in response to temperature and diet: Alessandra Schnider, Katja Räsänen, Kasha Strickland, Joseph S. Phillips, Bjarni K. Kristjánsson; Paper.

III. Changes in allometry and gene expression in threespine stickleback (*Gasterosteus aculeatus*) in response to temperature and diet: Alessandra Schnider, Bjarni K. Kristjánsson, Katja Räsänen, Kasha Strickland, Zophonías O Jónsson; Paper

Authors contributions

Table 1: Contribution of all co-authors of the papers (marked by I-III) included in this thesis and committee members who supervised and approved this thesis.

Author	Conceptualisation	Funding acquisition	Data acquisition	Analysis	Project management	Writing – original draft	Writing – review & editing
Alessandra Schnider	I-III	I-III	I-III	I-III	I-III	I-III	I-III
Bjarni K. Kristjánsson	I-III	I-III			I-II		I-III
Katja Räsänen	II-III	I-III			II-III		I-III
Zophonías O. Jónsson	II-III	I-III			III		I-III
Kasha Strickland							II-III
Joseph S. Phillips							II
Franka Hemme			I				I
Etienne de la Burgade			I				I
Blake Matthews							I-III
Catherine Peichel							I-III

Acknowledgements

This PhD project was made possible by the Icelandic Centre for Research Rannis though their financial support first through the grant of excellence (Nr. 195571-052) and the doctoral student grant (Nr. 228501-051). I also thank the University of Iceland for awarding me their teaching assistant grant which allowed me to continue my PhD project and to gain invaluable experience as an academic teacher. I would also like to thank the Nature Research Centre at Mývatn for their support and all the landowners around Lake Mývatn for allowing us to fish on their land.

Thanks to the opponents Lisa N. S. Shama and Antti P. Eloranta for accepting to evaluate this thesis.

I extend my deepest gratitude to my outstanding committee members. I had heard many a story of challenging committee members and I am beyond grateful that my story is one of support and cooperation. I was blessed with three completely different supervisors that, each in their own way, made this project the success it is, and me a better scientist thanks to their guidance. To my first supervisor Bjarni K. Kristjánsson, thank you for sharing your extensive knowledge of Icelandic freshwaters, history, customs, and beer. You always had an open door and ear for when I needed your support. Thank you for sharing your time and patience so generously, God knows it was necessary during what felt like a never-ending plasticity experiment. I also thank my second supervisor Katja Räsänen who, even though being an ocean away for most of this project always felt close. You pushed me to go the extra mile and allowed me to grow beyond the scope of this project. Third, but not least, to Zophonías O. Jónsson, thank you for welcoming you in your lab space and for guiding me through the expansive land of gene expression and university administration. I appreciate your no-nonsense communication sprinkled with all the sarcasm possible. To, Blake Matthews, thank you for lending your wit and inspired thinking to this project and for that most exquisite peanut butter sauce. Finally, I want to thank Catherine Peichel, the external committee member, for being so supportive and accessible. I am especially grateful that you freely shared your crossing protocols and expertise when I was struggling to cross a second generation.

To Kasha Strickland and Joe Phillips, thank you both for helping me take care of the fish on weekends, for all your statistical support and for all the impromptu meetings where I got to pick your brains. And to Kasha, thank you for being such a strong and passionate collaborator and for showing me that vulnerability and compassion at the workplace are strengths. You exemplifying what is possible when women uplift each other.

To the most amazing technician, Stephen Price, thank you for cultivating Cladocera for a whole two year for me and for developing all the rearing and sampling protocols with me. Also big thanks to Rakel Þórbjörnsdóttir for being the honorary stickleback teammate and helping even if it was not your job and for letting me vent whenever I needed it. We did not release Willy, but we sure had a good time sampling all those juveniles.

I would also like to thank the interns Quentin Amerigo, Line Lindholm, Aurore Dumont, Etienne de la Burgade, Arthur Bernardi, Luca Perisse, Franka Hemme, Nanda Vo without whom the plasticity experiment would not have been possible. Each of you also taught me a lesson in how to become a better mentor and I am immensely grateful for your hard work. A special thanks to Amber Monroe who jumped in when the COVID19 pandemic threatened to crush our experimental plans. Without your support and hard work, we would never have gotten past the first month.

Kári H. Árnason, our station manager and in house psychologist. Thank you for trusting me around the workshop, for all the treats, long car rides and talks. In short, thank you for being my friend. Not sure I would have made it this far without your unwavering support and pep talks.

To Camille Leblanc, thank you for being my sounding board and all the talks about work and life over the past five years. Your kind words and dedication to everything you do inspires me to do better myself. You and Alina have become family and made Iceland feel like home. Merci à vous deux pour tout!

To my friends Liliane Miotti, Angela Hüppi, Karin Troxler, Fabienne Romani, Sergio Roth, Marcel Ottiger, Celia Nosetti, Christian Zürcher, Andreas Hauser and Michael Muggli. Our weekly (online) pub quiz gatherings kept me sane during the Covid19 pandemic. Every care package brought a little bit of home to me and whenever I am back home you welcome me with open arms and make me feel like I never left. Ech cha ned en Wort fasse wie vell mer das bedütet!

Manuela Bizzozzero is the reason I even considered applying to this project. Although you sent me off to the cold north, you have remained a supportive friend throughout all these years. Thank you for being you, and can't wait to congratulate you on your own PhD!

To my sister, Loredana Schnider, you are my rock and have always stood by me. Words cannot capture the gratitude I hold for you and of course, thank you for all the care packages. Ech be stolz dini Schöster zsi. Ti amo sorellina!

Finally, my dearest Tryggvi Pálsson, thank you for your endless patience, for your incredible trust in my abilities and for believing in me when I doubted myself. I cannot wait to see what else life has in store for us!

1 Synopsis

1.1 Introduction

It is becoming increasingly clear that evolutionary and ecological processes can act on a comparable timescale (Hairston et al. 2005; Fouqueau and Polechová 2024; Kumawat et al. 2025). Therefore, it is crucial to take an integrative approach by consolidating ecology, evolutionary and developmental biology (Eco-Evo-Devo) to elucidate the evolutionary forces which give rise to, maintain, and alter biological diversity (Skúlason et al. 2019). Special focus should be put on uncovering the drivers that generate and maintain phenotypic variation, because the phenotype is the direct target of natural selection and therefore the foundation for evolutionary processes in response to selection. An individual's phenotype results from the interaction between its genes and both its current and past environments, including developmental and parental influences. (reviewed in Houle et al. 2010; Danchin et al. 2011). The understanding that the phenotype results from both genetic and environmental influences challenged the notion that genes have a fixed, unchanging effect on the phenotype. Consequently, phenotypic variation is not entirely explained by genetic variation: what used to be considered unwanted noise in the data has become the subject of interest (West-Eberhard 1989; West-Eberhard 2003; Pfennig et al. 2010). The collective research done to this day and technological advancements have made it possible to connect the dots in a more holistic fashion and to approach questions regarding phenotypic variation from a broader point of view. This will enable us to better understand the development of the phenotype and how it interacts with the environment, as well as the potential production of phenotypic variation which is ultimately the raw material for natural selection to act upon (Sultan and Stearns 2005).

The ability of a single genotype to produce different phenotypes in response to different environmental conditions is referred to as phenotypic plasticity (West-Eberhard 1989; Schlichting et al. 1998; Agrawal 2001; Garnas 2018). Phenotypic plasticity can allow organisms to adjust their phenotype to the present environment without relying solely on genetic changes. An array of studies has established that an individual’s phenotype can be shaped by the environment throughout its lifetime (Skúlason et al. 2019). Phenotypic plasticity can be divided into three categories: developmental, adult, and transgenerational plasticity (Pfennig 2021). Developmental plasticity is the ability of an organism to alter its developmental processes in response to environmental conditions and thereby change the phenotypic outcome (West-Eberhard 2003; Moczek et al. 2011). For instance, tadpoles of the spadefoot toads (*Spea multiplicate*) develop into two different ecomorphs based on their diet which is largely determined by availability of suitable prey (Levis et al. 2015). Developmental plasticity might be favourable when conditions change between generations but are stable within. Second, plasticity can occur in adult individuals, such as changing phenotypes in response to seasonality (e.g. fur colour, diet) or plasticity in traits central to reproduction (e.g mating strategy or breeding colouration) (West-Eberhard 1989; Ferreira et al. 2020). For instance, crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) possess the ability to modulate gill surface structure depending on

temperature and oxygen levels (Sollid and Nilsson 2006). In cold and normoxic conditions the space between the lamellae is filled up. Whereas warm temperatures and hypoxic conditions induce apoptosis in the interlamellar cell mass causing the lamellae to protrude and thus increasing the surface for oxygen uptake.

Finally, transgenerational plasticity encompasses the phenotypic variation in the offspring caused by the environment experienced by a parent (Mousseau and Fox 1998a; Danchin et al. 2011). For example, parental thermal environment has been found in various studies to affect not only the current generation but offspring metabolism and growth for instance (Salinas and Munch 2012; Shama 2015; Massey and Dalziel 2023). Considering that increased body size in fish generally leads to increased survival and higher fecundity, transgenerational effects can have major fitness implications (Sogard 1997; Wootton 2012). Consequently, phenotypic plasticity enables organisms to rapidly adapt to environmental variation both within and across generations. Thus, transgenerational effects underscore the importance of understanding how organisms allocate resources to maximize fitness, a concept central to life history theory. Indeed, life history can provide a valuable framework to understand phenotypic variation in response to varying environmental conditions and thus further our understanding of phenotypic plasticity.

Life history theory is based on the assumption that individuals are limited in their resources and therefore need to trade-off between allocating energy to growth, maintenance or investment into current versus future reproduction in order to maximise lifetime fitness (Stearns 1998; Kaplan and Robson 2009). An individual's life history is defined by crucial life events (e.g. birth, age at maturation, and death) and sensitive periods (e.g. development and reproduction). Therefore, to understand the fitness consequences of varying phenotypes it is important to consider life-history theory. Plasticity in traits linked to reproductive investment, such as clutch and egg size, are especially interesting, as these traits not only affect the parent's fitness, but can also shape the offspring's fitness via transgenerational effects (Ernsting and Isaaks 2000; Shama 2015). Life history theory can also help to explain why not all transgenerational effects are necessarily adaptive (Mousseau and Fox 1998b).

Selection acting on the parent maximises parental life-time fitness (Marshall and Uller 2007). In species that have multiple reproductive events, for example, selection might favour a decreased investment in current offspring in favour of increasing parental survival and the opportunity to reproduce later when conditions may be more favourable (Coleman et al. 1985; Mousseau and Fox 1998b; Losos et al. 2017). This can in some cases lead to a reduced offspring fitness due to smaller egg size or lower offspring quality (Marshall and Uller 2007). In such a case, transgenerational effects would not be adaptive but instead maximise the parent's lifetime reproductive success, thereby increasing its overall fitness. Therefore, transgenerational effects need to be set into context and examined carefully to understand their adaptive potential and the mechanism that mediate phenotypic plasticity within and across generations.

While there are many demonstrations of phenotypic plasticity within and across generations, the molecular mechanisms that mediate phenotypic plasticity are not fully understood yet. Variation in gene expression and RNA splicing have both been found to vary with changing environmental factors which can result in different phenotypes (Whitehead and Crawford 2006a; Whitehead and Crawford 2006b; Steward et al. 2022). For instance, the migratory locust (*Schistocerca gregaria*) occurs in two forms differing in

coloration, wing length and sociality. The two forms are mostly induced by population density and mediated through differential gene expression (Tawfik et al. 1999). Populations exhibiting considerable phenotypic diversity in the absence of genetic divergence are well-suited to test how contrasting environments can promote differential gene expression and how that in return manifests in plasticity in the phenotype.

Moreover, the effect of the environment on an organism is context dependent and is expected to depend on the life stage of an individual, as individuals at different developmental stages face varying challenges (Petitgas et al. 2013; Fernlund Isaksson et al. 2022). For example, diet is often a function of an individual's body size, resulting in distinct dietary patterns along developmental trajectories (e.g. ontogenetic niche shifts) (Amundsen et al. 2003; Kimirei et al. 2013). Dietary changes can also elicit differential gene expression (Panserat et al. 2009; Gunter et al. 2013). Consequently, gene expression can change drastically throughout development and between life stages. Furthermore, juveniles generally prioritise energy allocation to maximise growth, whereas sexually mature individuals divert energy away from growth in favour of reproduction (Heino and Kaitala 1999; Post and Parkinson 2001).

Furthermore, gene expression is tissue specific as organs have specialized functions and thus, may require different genes to be expressed (Singaraja et al. 2005). Changes in DNA-methylation, the addition or removal of a methyl group to cytosines in the DNA, can affect chromatin structure and can therefore alter the accessibility of a given DNA sequence for transcription, thus affecting gene expression. This mechanism can also affect the next generation if the DNA-methylation in germline cells is altered and survives reprogramming during germ cell- and early embryonic development (Danchin et al. 2011; Gilbert and Epel 2015; Matlosz et al. 2024). Interestingly, temperature has been found to impact methylation in early embryonic development, further stressing how dynamic the underlying machinery is that produces the observed phenotype (Matlosz et al. 2024). Thus, contrasting environmental cues can shape the phenotype through differential gene expression within and across generation.

1.1.1 Phenotypic plasticity in response to temperature

Temperature varies in space and time in natural ecosystems and temperature can have a great impact in shaping an ecosystem and the organisms living in it (Angilletta et al. 2006). As temperature is changing dramatically due to climate change, this may have serious consequences for reproducing and developing organisms (Merilä and Hoffmann 2016). Ectotherms, such as fish, are strongly impacted by temperature as they have limited means of regulating their body temperature. In fish, warm temperatures accelerate metabolic rate, growth, developmental rate, fecundity and age at maturation, which reveals substantial potential for phenotypic plasticity in relation to temperature (Clarke and Johnston 1999; Pörtner et al. 2001; Angilletta et al. 2004; Georga and Koumoundouros 2010; Ramler et al. 2014). Living in warmer temperatures increases the baseline energetic costs of an individual but can also accelerate physiological processes, allowing for faster development and earlier sexual maturation (Thunell et al. 2023). Generally, an increase in ambient temperature results in fishes maturing faster and at a smaller size. This has major fitness implications, as for instance female body size is generally highly correlated with egg number and size, which in turn is correlated with offspring survival and thus fitness (Mousseau and Fox 1998a; Heath et al. 1999).

At “optimal” temperatures, organisms thrive, and fitness can be maximised, whereas fish in “tolerable” conditions may survive, but at a fitness cost (Mariu et al. 2023). The respective optimal and tolerable temperature range is further subject to variation between and within species. For instance, a study on threespine stickleback (*Gasterosteus aculeatus*) from the Golf of Bothnia, Sweden, showed temperature tolerance as low as 4°C and as high as 30°C, but in experimental conditions growth showed a sharp peak at 21°C (Lefébure et al. 2011). Furthermore, while Arctic charr (*Salvelinus alpinus*) from central Canada showed normal heart function at 4°C up to 16°C, with the function rapidly deteriorating and failing at 21°C (Gilbert et al. 2020), Arctic charr from Greenland already became thermally limited at 13°C and exhibited heart failure at 15°C (Hansen et al. 2017). Thus, understanding the species and population specific context is essential to interpretation of any observations made. Large increases in water temperature can result in more severe stress responses, such as the accumulation of oxidative damage products (Carney Almroth et al. 2015). Furthermore, extreme temperatures can have deleterious effects on ovulation, fertility and egg survival (Boulé and Fitzgerald 1989; Pörtner et al. 2001), with subsequently strong effects on reproductive fitness. This suggests that physiological processes may be severely hampered beyond a certain increase in ambient temperature, resulting in reduced accuracy and efficiency (Alfonso et al. 2021). This is supported by various studies observing the upregulation of specific families of genes, such as heat shock proteins, interleukins, glutathione peroxidase and ion transporters, in an attempt to mitigate the harmful effects of increasing ambient temperatures (Islam et al. 2022).

Finally, temperature not only affects the development and performance of individuals in the present environmental circumstances but can also have an effect across generations. Many studies report transgenerational effects in which the thermal environment experienced by the parents affects the optimal developmental temperature of the offspring (Salinas and Munch 2012; Shama and Wegner 2014; Massey and Dalziel 2023). Furthermore, temperature effects can last across multiple generations (Shama and Wegner 2014).

1.1.2 Phenotypic variation in response to diet

Another important environmental factor shaping an organism's phenotype is its diet. Diet can be especially influential during the development of an organism, when the phenotype tends to be most plastic (West-Eberhard 2003), and thus may substantially impact the adult phenotype (Pfennig 2021). The effect of diet can be as drastic as in honey bees (*Apis mellifera*) where larvae fed with “royal jelly” (especially rich in proteins) will develop into queens whereas larvae raised on beebread (a mixture of pollen and nectar) will develop into workers (Kamakura 2011; Zhu et al. 2017). Another example can be found in fish, especially cichlids, where jaw, skull and whole body shape can be altered by diet (Wimberger 1991; Wimberger 1992). These examples illustrate how diet can drastically alter morphology, behaviour and reproductive strategies and unveils the great potential for phenotypic plasticity in response to diet.

The development of the feeding apparatus in fish is often directly impacted by diet and shows extensive potential for plasticity (Day and McPhail 1996; Hegrenes 2001; Crichigno et al. 2014). For instance, fish feeding on a benthic diet tend to have a wider jaw and shorter gill rakers, whereas individuals feeding on a pelagic diet have a more narrow-

angled jaw and longer gill rakers (seen in e.g. *Cichlasoma managuense* (Meyer 1987), threespine stickleback (Day and McPhail 1996), Orangespotted sunfish (*Lepomis humilis*) (Hegrenes 2001), European whitefish (*Coregonus lavaretus*) (Amundsen et al. 2004), *Percichthys trucha* (Crichigno et al. 2014). Moreover, different diets may require different foraging strategies and therefore varying body shapes may be advantageous (Mérigoux and Ponton 1998). Furthermore, depending on the microenvironment the available diet items may vary and, therefore, diet induced morphological changes are not only a direct reflection of food consumed but can also reflect the environment the individual inhabits. Nevertheless, there are also limitations to plasticity. Many dietary adaptations have a strong genetic component, as for example, the reduction of gill rakers in freshwater stickleback, compared to their marine ancestor, has been found to have a strong genetic component (Glazer et al. 2014).

Moreover, as for temperature, also an individual's diet not only affects its own phenotype but can also extensively impact its offspring's phenotype (Steinberg 2018). Maternal diet can greatly influence the development of an embryo (Mousseau and Fox 1998a). In species with internal gestation, such as mammals, viviparous fish and some reptiles, signals from the mother's diet can be passed directly to the embryo. Likewise, in species where eggs mature externally, the mother's environment can influence the embryo through variation in amount and composition of egg yolk (Krist 2011). For instance, a study on threespine stickleback from Paxton Lake, British Columbia, found that limnetic individuals had a higher variability in their diet, compared to benthic individuals (Schluter 1993). A laboratory experiment on this population then found that offspring from limnetic parents showed greater plasticity in response to diet than did offspring from benthic parents (Day et al. 1994). Therefore, it is evident that diet induced phenotypic plasticity, within and across generations, can contribute substantially to phenotypic variation.

1.2 Aims and objectives of thesis

The aim of this PhD thesis is to further our understanding of the role phenotypic plasticity may play in shaping the phenotype within and across generations, specifically in response to temperature and diet. The transgenerational aspect is central to this project, as experiments with realistic temporal variability are still rare. Also, studies have tended to investigate the effects of environmental drivers of phenotypic variation in isolation. Thus, despite extensive studies on the long-term effects of elevated temperatures and varying diets on individual performance, not much is known so far of their interaction and their potential transgenerational effects. Finally, I also aim to understand some of the underlying mechanisms of phenotypic plasticity, with a special focus on maternal effects and gene expression patterns. This thesis approaches these aims in the following the papers, using threespine stickleback of Lake Mývatn, Iceland:

I) Juvenile feeding ecology of threespine stickleback
In this first paper I studied the feeding ecology (e.g. diet, head morphology, and correlation between diet and morphology) of wild caught juvenile threespine stickleback. This is one of the first studies to investigate how the diet of juvenile freshwater stickleback relates to morphology in a wild setting. It allowed me to understand what patterns are observed in the wild and thus, contextualise the experimental results in a more meaningful manner.

II) Plasticity in key life history traits in threespine stickleback in response to temperature and diet
In this second paper I tested the effects of temperature and diet on key life history traits (e.g body size, reproductive investment, and hatching success) across multiple developmental stages (e.g. embryonic, juvenile, and sexually mature) within and across generations.

III) Changes in allometry and gene expression in threespine stickleback in response to temperature and diet
In the third paper I tested the effects of temperature and diet on body size, body condition and allometry (i.e. relative head size) and differential gene expression in livers of sexually mature threespine stickleback in response to contrasting temperatures and diets.

1.3 Study system

1.3.1 Threespine stickleback

Since the last ice age 10'000-14'000 years ago marine threespine stickleback have repeatedly colonized a wide variety of freshwater bodies across the northern hemisphere (Bell and Foster 1994; Schluter 2003). Threespine stickleback have served as model species to study rapid adaptive radiation (Schluter 2003; Hendry et al. 2013), where diverging morphs or ecotypes along axes of ecologically divergent environments have been described in depth (Robinson 2000; Kristjánsson 2001; Hendry et al. 2002). Most commonly overall body shape, feeding morphology (e.g. gill raker number and morphology) and defence traits (e.g. armour plate number and spine length) have been connected to environmental drivers such as salinity, predation, temperature and diet, and commonly described along the benthic-limnetic axis (Hendry et al. 2002; Kristjánsson et al. 2002; Bolnick et al. 2008; Berner et al. 2010; Ravinet et al. 2013).

Stickleback feeding on a benthic diet tend to have a wider jaw with fewer and shorter gill rakers, and a sub-terminal facing mouth and overall larger body size (Day et al. 1994; Cresko et al. 2007). In contrast, individuals feeding on a limnetic diet tend to have a narrower jaw with more numerous and longer gill rakers, and a more terminal facing mouth and smaller body size (Day et al. 1994; Robinson 2000; Cresko et al. 2007; Crichigno et al. 2014). Complementary laboratory studies have found that this divergence has a strong genetic component but can as well be facilitated by phenotypic plasticity, which is known to be common in threespine stickleback (Wund et al. 2008). However, morphological studies in stickleback have heavily focused on the fully formed adult phenotype (Day and McPhail 1996; Cresko et al. 2007; Garduno-Paz et al. 2010). As the phenotype develops throughout the juvenile life stage the adult phenotype cannot be fully explained by the current environment an individual lives in. Therefore, we need to look at individuals at varying life stages and their respective ecology.

The relatively small and fully sequenced threespine stickleback genome has made stickleback a great organism to study the genetic foundation for phenotypic diversity (reviewed in Cresko et al. 2007; Peichel and Marques 2017; Reid et al. 2021). Specifically, gene expression studies across various environmental stimuli (e.g. temperature, diet, predation and social interactions) have furthered our understanding of the underlying molecular mechanisms involved in phenotypic plasticity and adaptation in stickleback (Greenwood and Peichel 2015; Metzger and Schulte 2018; Reid et al. 2021). For example, one study found that more than 5000 genes displayed similar plasticity in gene expression in both marine and freshwater sticklebacks of British Columbia, but freshwater populations showed a significantly higher number of genes with plastic expression compared to marine populations when reared near thermal tolerance extremes in the laboratory. This observed plasticity in gene expression could have facilitated the repeated colonialization of freshwater habitats by stickleback (Morris et al. 2014).

Another gene expression study using threespine stickleback from the River Ulla, Spain, showed that changes in gene expression related to metabolism, homeostasis and signalling pathways were plastic in response to increasing temperatures (Kim et al. 2017). This allowed individuals to manage thermal stress but simultaneously individuals experienced a

reduction in fitness. Furthermore, gene expression patterns have been found to differ between sexes both at juvenile and adult life stages (Kitano et al. 2020). Differences in gene expression can also be found across generations, whereby for instance maternal stress elicited differential gene expression in sons but not daughters (Metzger and Schulte 2016). Furthermore, mitochondrial gene expression has been connected to changes in physiology in response to contrasting temperatures, stressing the impact of maternal effects, especially, in differential gene expression (Shama et al. 2016).

There is also a growing body of evidence from multigenerational experiments that parental and grandparental environment can affect offspring phenotype in threespine stickleback (Shama et al. 2014; Shama and Wegner 2014; Heckwolf et al. 2018; Hellmann, Bukhari, et al. 2020; Hellmann, Carlson, et al. 2020). These transgenerational effects have often been connected to a variation in parental investment between contrasting environmental conditions (Candolin 1998; Poizat et al. 1999; Baker et al. 2015). Furthermore, several life history traits, such as size at maturation and clutch size, have been shown to be plastic in response to contrasting environmental conditions, which stresses the importance of parental and grandparental transgenerational effects in this species specifically (Shama et al. 2014; Shama and Wegner 2014; Baker et al. 2015). Thus, threespine stickleback are an excellent system to study phenotypic diversification and the potential for within and across generational plasticity.

1.3.2 Lake Mývatn

Mývatn (65°36'N, 17°00'W; 278m a.s.l.) in north-eastern Iceland is a shallow eutrophic lake, that varies greatly in a range of abiotic and biotic factors over space and time (Einarsson et al. 2004) (Fig. 1.1). Its 37km^2 area is divided into two main basins connected by two narrow channels: a smaller, spatially more heterogeneous and deeper (1-6m depth) north basin (Ytri flói in Icelandic) and a more homogenous and shallower (2-3.2m depth) south basin (Syðri flói in Icelandic) (Einarsson and Örnólfsdóttir 2004; Phillips et al. 2023). The lake is mostly fed by springs along its eastern shore, ranging from cold water springs (~5°C) in the south-east to geothermal hot springs (up to ~23°C) in the north-east, creating a temperature gradient on the eastern shoreline from the "Warm" habitat (from here on "Hot Shore") in the north-east, with average year-round water temperature of 20-23°C, to a constantly cold (~5°C) habitat in the south-east, which is generally uninhabited by threespine stickleback (Phillips et al. 2023). The rest of the lake's water temperature fluctuates with seasonal air temperature with average summer temperatures ranging from 11-13°C (Ólafsson 1979; Millet et al. 2013). The lake has commonly a barren rocky shoreline that drops off into a deeper vegetated. Such a shore habitat can for example be found in the south-west of the lake (from here on "Cold Shore").

The lake further greatly varies in productivity and invertebrate abundance and community structure in space and time (Einarsson and Örnólfsdóttir 2004; Ives et al. 2008; Bartrons et al. 2015). Consequently, Mývatn stickleback experience great seasonal fluctuations in temperature as well as variation in food availability and prey composition across years (Ives et al. 2008; Bartrons et al. 2015; Phillips et al. 2023). Studies on this stickleback population have shown that diet composition varies across habitats and seasons, with individuals feeding predominantly on Chironomidae in early summer but in some areas shifting to a more Cladocera based diet later in the season (Adalsteinsson 1979; Millet 2013). Indeed, the trophic morphology of adult stickleback, such as gill raker structure and

gut length, as well as diet composition have been found to be associated with contrasting habitats, and difference in prey availability within and across seasons and years (Millet 2013; Snorradóttir 2023; Strickland et al. 2024). Stickleback inhabiting contrasting habitats further show considerable phenotypic divergence (Kristjánsson et al. 2002; Ólafsdóttir et al. 2007; Millet et al. 2013; Strickland et al. 2023). However, while an earlier studies reporting strong genetic divergence (Ólafsdóttir et al. 2007), more recent studies found only weak genetic divergence (Millet 2013; Strickland et al. 2023).

Furthermore, in large population-wide events the numbers of threespine stickleback in Mývatn rise and fall in a semi-cyclic fashion with source-sink connection between the Southern basins, where density is often low and the much higher stickleback density in the Northern basin (Phillips et al. 2023). Consequently, the population may potentially undergo a genetic bottleneck event with each population crash. Still a genetic study on this population found no evidence to suggest low genetic variation (Strickland et al. 2023). Major environmental variables experienced by stickleback in the lake are large temperature gradients, varying food items and different predators and predations pressure (Strickland et al. 2023). However, not much was known about the juveniles in this population, and I could only find few studies that had characterised the diet of wild juvenile stickleback living in

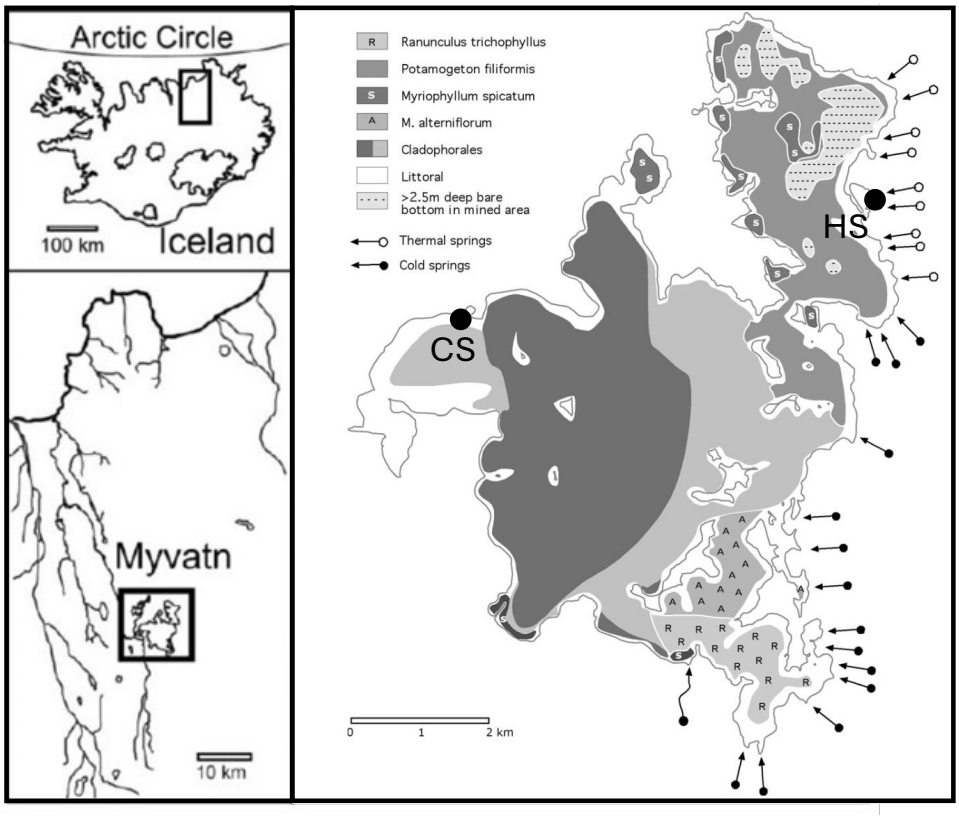

Figure 1.1 *Map of Lake Mývatn, NE Iceland, depicting the dominant benthic macrophytes (in late 2000) and the main spring water inflows. The two shades of grey used to describe the Cladophorales habitats reflect density (lighter: less dense, darker: denser). Black circles mark the two sampling sites of threespine stickleback representing the two habitats "Cold Shore" (labelled "CS") and "Hot Shore" (labelled "HS"). Edited from Einarsson et al. (2004).*

Freshwater (Hynes 1950). This was a gap in our knowledge, considering that numerous laboratory experiments had been conducted to test the effect of diet on the developing phenotype of juvenile stickleback (Day and McPhail 1996; Garduno-Paz et al. 2020). From those laboratory studies we know that threespine stickleback show varying levels of developmental plasticity in their body shape, head morphology and gill raker number and size in response to diet (Day et al. 1994; Garduno-Paz et al. 2010; Ramler et al. 2014; Shama and Wegner 2014), including a laboratory experiment on Mývatn stickleback (Delarue 2016).

1.4 Main results and discussion

How biological diversity arises and is maintained is still the centre of scientific inquiry. In this thesis I specifically address phenotypic variation in response to temperature and diet across generations. I chose Lake Mývatn for this study as this is a highly dynamic system and the local threespine stickleback population exhibits phenotypic divergence along multiple ecological axis. In **Paper I**, I described the juvenile diet and head morphology of threespine stickleback of Lake Mývatn and showed that diet was correlated with head morphology across the various habitats encountered in Mývatn. I further found a dietary niche shift with increasing body size, as individuals preyed upon bigger and more varied prey items with increasing body size. The major dietary groups consumed by juveniles were Cladocera and midge larvae. Previous studies show that these are also the major dietary groups in adult threespine stickleback (Millet 2013; Snorradóttir 2023). Thus, in my experiment, which was the basis for **Paper II** and **Paper III**, I fed contrasting diets consisting of Cladocera (epibenthic) or midge larvae (benthic), aimed at recreating the major dietary contrasts of both juveniles and adults found in nature.

The plasticity experiment, in which I reared threespine stickleback across three generations in contrasting environmental conditions, was the foundation for my second and third paper. It was important for me to run the experiment across a more realistic temporal scale and environmental variability as such experiments are rare. Therefore, I tested the effects of contrasting temperatures (cold: 12°C vs. warm: 22C°) and diets (benthic vs. epibenthic) on various phenotypes within and across generations of juveniles and adult individuals. I also tested for parental/grandparental origin effects of the two contrasting habitats (“Cold Shore” vs. “Hot Shore”), where the wild parental fish (P) originated from, which contrast in thermal conditions and prey availability among others. This allowed me to test for adaptive divergence between habitats and to assess if the observed differences in the wild are due to genetic or plastic effects.

In **Paper II** I focused on the effect of contrasting temperatures and diets on key life history traits such as juvenile size, size at sexual maturation and maternal investment (i.e. clutch and egg size), and reproductive success (i.e. hatching success). I studied individuals at different developmental stages across multiple environmental factors, as often studies focus on single developmental stages and single environmental factors, which neglects the potential effect early life experiences might play later in life. Furthermore, by measuring reproductive success I have a direct fitness measurement, which allows me to make more direct interpretations about the effects of contrasting environmental conditions on key life-history traits. Finally, by comparing phenotypic variation across developmental stages or

even generations allow me to further our understanding of phenotypic plasticity in this system.

1.4.1 Temperature and diet effects of key life history traits

The results in **Paper II** show that both temperature and diet affected key life history traits within and across generations. However, I observed varying patterns in phenotypic variation across generations and found some intriguing results. Juveniles of the first experimental generation (F1) grew bigger on a benthic diet and in cold rearing conditions, whereas in the second generation (F2) juveniles on an epibenthic diet grew bigger irrespective of parental diet. This inversion of patterns was quite surprising and not anticipated and I still have no explanation for the causes of this. Another surprise came when body size pattern of the adult F1 generation were examined. Individuals grew bigger in warm rearing conditions, but possibly one of the strongest signals was a stark reduction in hatching success in the offspring of F1 adults in warm rearing conditions compared to the cold (Fig. 3.3 **Paper II**). I had not anticipated this, as I had found no difference in the hatching success in the offspring from the wild parental individuals (Table 3.4, **Paper II**).

The average temperature in warm habitat in Mývatn had been reported to be 23° which is higher than our experimental temperatures (Millet 2013). Furthermore, through personal communications I knew that stickleback males were building nests in these waters and I have myself observed numerous juveniles by the spring inlet, indicating reproductive activity. To verify previous temperature measurements, I placed temperature loggers in Hot Shore, with increasing distance from the hot water springs as well as at Cold Shore, with increasing distance from the shore (June 2022 – October 2023). I learned that the temperatures in HS was stable at 23°C only at the spring outlet. The further away from the spring the higher the daily temperature variation was, but more importantly the mean temperature decreased, and was closer to 18°C. Whereas in Cold Shore the temperatures were more stable across the habitat and daily fluctuations were low (Fig. 1.2).

Consequently, the constant 22°C simulated in the laboratory experiment only reflects few spots (spring outlets) in Hot Shore and might not be the preferred nesting habitat. Potentially, males only nest near the hot spring inlets in Hot Shore because they were not able to compete for more desirable locations. It would be interesting to explore if females do choose to mate with these males and to compare reproductive phenotypes (e.g. breeding colouration, nesting behaviour and reproductive success) among males across Hot Shore. Further one could look at females across the Hot Shore habitat and test if breeding condition (e.g. body size and condition and age) and maternal investment (e.g. clutch size, egg size and within clutch egg size variation) varies in response to distance to the hot spring inlet and if such differences do correlate with reproductive success.

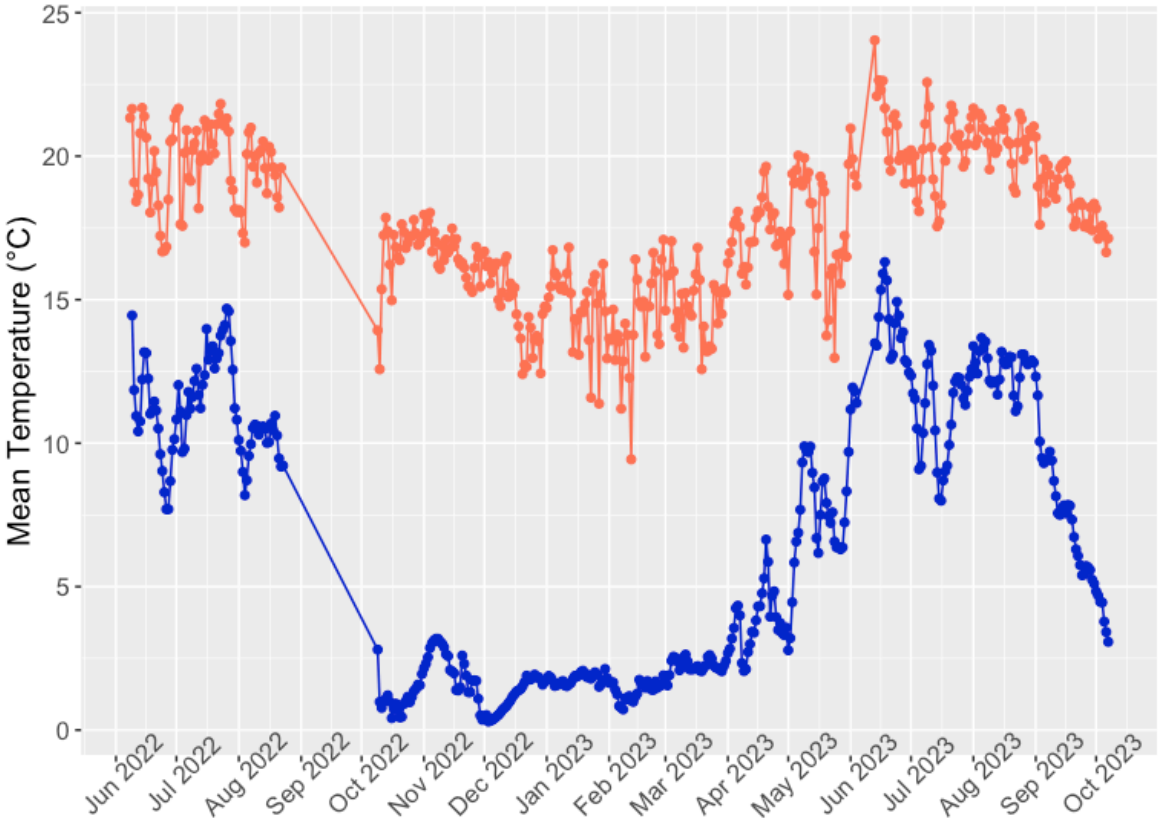

Figure 1.2 *Mean daily temperatures (in °C) of the two habitats Cold Shore (blue) and Hot Shore (orange) in Lake Mývatn, northern Iceland. Between June 2022 and October 2023 four temperature loggers were deployed along the shore area. In Hot Shore a hot spring feeds into the lake, therefore, the loggers were placed at the inlet of the spring and at increasing distance from it. Gaps in temperature records in autumn 2022 and spring 2023 were due to extracting data from the logger and replacing the batteries.*

1.4.2 Paternal investment and transgenerational effects

Preliminary results from our rearing experiment reveal that males in the warm temperature treatment showed brighter breeding colouration that covered a wider area than males in the cold (Schnider unpublished). Our preliminary results also indicate that breeding colouration was positively correlated with hatching success (Schnider unpublished). However, males in warm rearing conditions also had a lower body condition index than males in the cold (**Paper III**). Moreover, genes involved in metabolism, especially lipid metabolism, were upregulated in liver of males from the cold temperature in both the lab and the wild (**Paper III**). Stickleback store energy in the form of fat in the liver, thus a higher lipid metabolism in the liver indicates greater energetic resources (Chellappa et al. 1995). Together with the decreased hatching success in the warm rearing conditions, this suggests that breeding colouration is rather an honest signal of reproductive investment rather than overall male condition.

Comparable patterns in breeding colouration have been observed in a study on threespine stickleback where resource restricted males showed brighter breeding colouration compared to males that were not restricted in their resources (Mehlis et al. 2015). Furthermore, starved males had a higher mortality rate, smaller body size and lower sperm

count. A similar study in pygmy halfbeaks (*Dermogeny colletti*), found the same pattern in breeding colouration between males fed a restricted and high-quality diet (Fernlund Isaksson et al. 2022). Additionally, that study found that resource restriction during development had similar negative effects in reproductive traits as resource limitation after reaching sexual maturity, further stressing how sensitive the developmental stages can be. In my experiment I observed an increase in mortality rate and fungal infections during the reproductive phase in warm treatment conditions (Schnider unpublished), indicating that the warm temperatures exacerbate physiological stress and weaken immune response. In warm reared adults I also found an increase in the expression of genes associated with immune response, further supporting that the warm rearing conditions imposed an additional challenge (**Paper III**). Thus, it appears that males in the warm are prioritizing current reproduction, whereas males in the cold temperature treatment might be prioritizing future reproductive events as well.

1.4.3 Maternal investment and transgenerational effects

Females also showed a clear response to contrasting rearing conditions in their maternal investment (**Paper II**). In the cold and on an epibenthic diet females had fewer but bigger eggs. Additionally, within clutch egg size variation was also lower for the cold reared females compared to warm reared females (Schnider unpublished). Increased variation in egg size may be adaptive as a bet hedging strategy appropriated by individuals in environments with unpredictable conditions (Shama 2015). From my long-term temperature measurements, I know that the daily temperature fluctuation in Hot Shore is much greater compared to Cold Shore (Fig. 1.2). Thus, the incubation time might vary greatly depending on current temperatures. Consequently, in Hot Shore having a variation of egg sizes may maximise a female's reproductive success. Transgenerational effects of origin within Lake Mývatn were strongest on hatching success in the warm rearing conditions. Clutches from a Hot Shore lineage showed a higher hatching success in warm rearing conditions, which could indicate an adaptation to reproduction in elevated temperatures. A genomic study on Mývatn stickleback did find loci associated with kidney function and immune system were enriched in relation to temperature (Strickland et al. 2023). This could mean that females with a Hot Shore origin may tolerate warm rearing conditions better and thus, be able allocate more energy towards reproduction. Thus, there could be a genetic basis for this effect rather than transgenerational plasticity.

Furthermore, the results from my experiment suggest that an epibenthic diet was less favourable in comparison to a benthic diet as individuals on an epibenthic diet grew less and had a lower body condition compared to individuals on a benthic diet (**Paper II**). Moreover, wild juveniles shifted to a more benthic diet with increasing body sizes, further supporting the hypothesis that this is a more favourable diet (**Paper I**). Therefore, Females producing bigger eggs on an epibenthic diet might be able to buffer a perceived suboptimal food availability by giving their offspring a head start in size at hatching, by providing more nutrition per individual by modulating egg size. In my experiment I did find that offspring of mothers fed an epibenthic diet were indeed bigger as juveniles than offspring of mothers on a benthic diet, supporting an environmental buffering scenario, where maternal investment helps mitigate the effects of warm temperatures and an epibenthic diet. Thus, this second paper highlights the impact transgenerational effects can have on fitness and the importance plasticity can play in key life history traits. It also shows the importance of multigenerational experiments. Had I ended this experiment at F1 adult size without any measurement of fertility or reproductive success I would have drawn very

different conclusions compared to what I concluded now, based on the knowledge I have gained by running the experiment for an additional generation.

1.4.4 Body allometry and differential gene expression in contrasting environments

The aim of the third paper was to study gene expression patterns and the potential energetic challenges imposed by contrasting temperatures and diets by studying overall body size and condition as well as allometry, the relationship of size to body shape, which can give insight into resource allocation during development (Post and Parkinson 2001; Olsson et al. 2007). To further understand the observed phenotypic variation, I tested for differential gene expression, the underlying mechanism for phenotypic plasticity, between temperature and diet treatments. To this end I took body size measurements and collected liver samples, a key organ in metabolic processes, of F1 males and females at sexual maturation. I further collected liver samples from wild sexually mature males and females from the two contrasting habitats used in the plasticity experiment (Cold Shore and Hot Shore), to analyse gene expression patterns in the wild that may be in response to environmental differences between habitats.

I found that allometry of F1 adults was affected by temperature, diet, and sex, as individuals in the warm rearing conditions and on an epibenthic diet had relatively bigger heads, indicating differential resource allocation during development and potential resource limitations. A study on Greenland threespine stickleback did connected resource limitation with relative head size (Moosmann et al. 2023). So, this could be a way to measure resource limitation during development across habitats and populations. I would further combine the measurement of relative head size with current body condition and liver size, as stickleback store fat in the liver, to gain a more short-term measurement of resource limitation.

From the gene expression data, I identified various differentially expressed genes in response to temperature associated with heat stress and immune response in experimental, wild individuals, and both sexes alike. As predicted the strongest response in gene expression was related to temperature, but I also found differential gene expression in response to diet in F1 females, whereas no diet-related differentially expressed genes were observed in F1 males. The pattern might be explained by males investing most of their time and effort into breeding, and often reduce or even cease to forage. Although I did not notice that males in the experiment stopped feeding, I did not systematically track behaviour and thus it is possible, that males did indeed reduce food intake. Alternatively, the difference in male and female gene expression may be due to sex-specific differences in metabolic regulation or hormonal influences. Moreover, I found that origin within Lake Mývatn had a negligible effect on gene expression patterns. The clear transgenerational effects of origin on hatching success further supports the hypothesis that there is a genetic component in the adaptation to reproduction in warm temperatures in this population (**Paper II**). In the future I aim to combine my list of differentially expressed genes with putative SNPs identified in a genomic study on wild Mývatn stickleback (Strickland et al. 2023) to further shed light on the mechanisms that shape the observed phenotypic variation.

In **Paper III** I gained insight into how potential energetic limitations can impact body allometry. Furthermore, I have found multiple differentially expressed genes in response to

temperature that could help mitigate environmental stressors, which would facilitate dispersal into novel habitats. This could be quite relevant for the threespine stickleback population of Lake Mývatn, which repeatedly undergoes major population level events marked by population crashes, followed by a build-up in numbers across habitats. Finally, differential gene expression studies integrating multiple environmental factors across a biologically relevant time frame add crucial information on how phenotypic plasticity can contribute to biological diversity.

1.5 Conclusions and outlook

This thesis explores the dynamic interplay of phenotypic plasticity and transgenerational effects in response to contrasting environmental conditions for shaping key life history traits. It further investigates the underlying mechanism of phenotypic plasticity through differential gene expression. By taking an integrative approach, I offer insights into the forces which produce, maintain, and shape biological diversity. I demonstrate that phenotypic plasticity can play a role in shaping key life history traits which are directly linked to fitness and thus adhere to the pressures of natural selection. Furthermore, contrasting environmental conditions not only impacted life history traits such as growth, maternal investment and reproductive success within a generation but also revealed the profound influence transgenerational effects can have.

Gene expression analyses complement these findings by exposing temperature-driven responses involving metabolic processes, stress tolerance and immune function. This project elucidates the underlying mechanism and importance of phenotypic plasticity, alongside the genetic basis of traits, in explaining observed biological diversity and thus, the persistence of populations in dynamic ecosystems. Considering the interaction between the environment, individuals, and populations as a whole, it is imperative to understand the broader forces that govern biological diversity.

My thesis further stresses the importance of multigenerational experiments under ecologically relevant conditions to fully capture the importance of phenotypic plasticity, within and across generations, for ecological and evolutionary processes. It also shows the value for more collaborative studies; thus, in the future, projects that combine physiology, ecology, genomics and evolutionary biology are indispensable. Studies that combine functional traits with underlying genotypes are labour intensive, but possible. Especially the growing number of long-term data sets offer invaluable opportunities. For example, the data produced in this PhD thesis will be combined with long-term data from the wild (Strickland et al. 2023; Strickland et al. 2024), phenotypic and genomic data alike, to better understand the genotype-phenotype-environment association across space and time.

The combination of ecological field data, with controlled laboratory and field experiments can bridge the gap between ecological and evolutionary perspectives. Thus, future research can continue to uncover the epigenetic mechanisms that regulate gene expression in response to varying environmental circumstances, such as temperature and diet. Moreover, as in this study, the interaction of multiple environmental variables ought to be prioritized as ecologically relevant patterns might only emerge in a more realistic context. Multigenerational experiments in a natural setting could pose such an opportunity. Future studies should further aim at combining data from multiple populations, or even species,

across a wide geographical and ecological range to understand the large-scale patterns that shape biological diversity.

1.6 Challenges

Traditionally this section is not included in theses at the University of Iceland, but I think it is important to acknowledge challenges overcome along the way. The global Covid19 pandemic was a big hurdle while I was setting up my experiment in April 2020. Due to the pandemic many of our supplies to build the brand-new system, which would house all my experimental fish, could not be shipped in time. This left us with many creative solutions to get everything up and running so I could start the experiment in June 2020. I also had to work with a much smaller team, as students were not allowed to travel, which made the beginning of the experimental phase additionally challenging.

Furthermore, working with a custom-built system meant that I had to develop and adjust protocols for rearing and sampling to fit my specific needs. Especially cultivating wild strains of Cladocera proved challenging, as they, despite stable laboratory conditions, showed fluctuations in productivity. As a result of that we had fluctuation in the supply throughout the experimental phase. An additional challenge was also my lack of experience with rearing fish, which made for a steep learning curve. However, when I initially failed to cross a second generation in the warm rearing conditions, I was fortunate enough that other labs, especially Catherine Peichel and her group, shared their experience and protocols with me. This incident further showed me the importance of published rearing protocols and sharing detailed information on long-term care of individuals.

References

Adalsteinsson H. 1979. Size and food of Arctic char *Salvelinus alpinus* and stickleback *Gasterosteus aculeatus* in Lake Mývatn. Oikos. 32:228.

Agrawal AA. 2001. Phenotypic plasticity in the interactions and evolution of species. Science. 294(5541):321–326.

Alfonso S, Gesto M, Sadoul B. 2021. Temperature increase and its effects on fish stress physiology in the context of global warming. J Fish Biol. 98(6):1496–1508.

Amundsen P-A, Bøhn T, Popova OA, Staldvik FJ, Reshetnikov YS, Kashulin NA, Lukin AA. 2003. Ontogenetic niche shifts and resource partitioning in a subarctic piscivore fish guild. Hydrobiologia. 497(1):109–119.

Amundsen P-A, Bøhn T, Våga GH. 2004. Gill raker morphology and feeding ecology of two sympatric morphs of European whitefish (*Coregonus lavaretus*). Ann Zool Fennici. 41(1):291–300.

Angilletta MJ Jr, Bennett AF, Guderley H, Navas CA, Seebacher F, Wilson RS. 2006. Coadaptation: a unifying principle in evolutionary thermal biology. Physiol Biochem Zool. 79(2):282–294.

Angilletta MJ Jr, Steury TD, Sears MW. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol. 44(6):498–509.

Baker JA, Wund MA, Heins DC, King RW, Reyes ML, Foster SA. 2015. Life-history plasticity in female threespine stickleback. Heredity . 115(4):322–334.

Bartrons M, Einarsson Á, Nobre RLG, Herren CM, Webert KC, Brucet S, Ólafsdóttir SR, Ives AR. 2015. Spatial patterns reveal strong abiotic and biotic drivers of zooplankton community composition in Lake Mývatn, Iceland. Ecosphere. 6(6):art105.

Bell MA, Foster SA. 1994. The evolutionary biology of the threespine stickleback. Oxford University Press.

Berner D, Roesti M, Hendry AP, Salzburger W. 2010. Constraints on speciation suggested by comparing lake-stream stickleback divergence across two continents. Mol Ecol. 19(22):4963–4978.

Bolnick DI, Caldera EJ, Matthews B. 2008. Evidence for asymmetric migration load in a pair of ecologically divergent stickleback populations. Biol J Linn Soc Lond. 94(2):273–287.

Boulé V, Fitzgerald GJ. 1989. Effects of constant and fluctuating temperatures on egg production in the threespine stickleback (*Gasterosteus aculeatus*). Can J Zool. 67(6):1599–1602.

Candolin U. 1998. Reproduction under predation risk and the trade–off between current and future reproduction in the threespine stickleback. Proc R Soc Lond B Biol Sci. 265(1402):1171–1175.

Carney Almroth B, Asker N, Wassmur B, Rosengren M, Jutfelt F, Gräns A, Sundell K, Axelsson M, Sturve J. 2015. Warmer water temperature results in oxidative damage in an Antarctic fish, the bald notothen. J Exp Mar Bio Ecol. 468:130–137.

Chellappa S, Huntingford FA, Strang RHC, Thomson RY. 1995. Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. J Fish Biol. 47(5):775–787.

Clarke A, Johnston NM. 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. J Anim Ecol. 68(5):893–905.

Coleman RM, Gross MR, Sargent RC. 1985. Parental investment decision rules: a test in bluegill sunfish. Behav Ecol Sociobiol. 18(1):59–66.

Cresko WA, McGuigan KL, Phillips PC, Postlethwait JH. 2007. Studies of threespine stickleback developmental evolution: progress and promise. Genetica. 129(1):105–126.

Crichigno SA, Battini MÁ, Cussac VE. 2014. Diet induces phenotypic plasticity of *Percichthys trucha* (Valenciennes, 1833) (Perciformes, Percichthyidae) in Patagonia. Zool Anz. 253(3):192–202.

Danchin É, Charmantier A, Champagne FA, Mesoudi A, Pujol B, Blanchet S. 2011. Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. Nat Rev Genet. 12(7):475–486.

Day T, McPhail JD. 1996. The effect of behavioural and morphological plasticity on foraging efficiency in the threespine stickleback (*Gasterosteus sp.*). Oecologia. 108(2):380–388.

Day T, Pritchard J, Schluter D. 1994. A comparison of two sticklebacks. Evolution. 48(5):1723–1734.

Delarue C. 2016. Plasticity of trait divergence in threespine stickleback (*Gasterosteus aculeatus*) from Lake Mývatn. Kristjánsson B, Räsänen K, editors. MSc Thesis, Hólar University.

Einarsson Á, Örnólfsdóttir EB. 2004. Long-term changes in benthic Cladocera populations in Lake Myvatn, Iceland. Aquat Ecol. 38(2):253–262.

Einarsson Á, Stefánsdóttir G, Jóhannesson H, Ólafsson JS, Már Gíslason G, Wakana I, Gudbergsson G, Gardarsson A. 2004. The ecology of Lake Myvatn and the River Laxá: Variation in space and time. Aquat Ecol. 38(2):317–348.

Ernsting G, Isaaks A. 2000. Ectotherms, Temperature, and Trade-offs: Size and Number of Eggs in a Carabid Beetle. Am Nat. 155(6):804–813.

Fernlund Isaksson E, Reuland C, Kahrl AF, Devigili A, Fitzpatrick JL. 2022. Resource-dependent investment in male sexual traits in a viviparous fish. Behav Ecol. 33(5):954–966.

Ferreira MS, Alves PC, Callahan CM, Giska I, Farelo L, Jenny H, Mills LS, Hackländer K, Good JM, Melo-Ferreira J. 2020. Transcriptomic regulation of seasonal coat color change in hares. Ecol Evol. 10(3):1180–1192.

Fouqueau L, Polechová J. 2024. Eco-evolutionary dynamics in changing environments: integrating theory with data. J Evol Biol. 37(6):579–587.

Garduno-Paz MV, Couderc S, Adams CE. 2010. Habitat complexity modulates phenotype expression through developmental plasticity in the threespine stickleback. Biol J Linn Soc Lond. 100(2):407–413.

Garduno-Paz MV, Huntingford FA, Garrett S, Adams CE. 2020. A phenotypically plastic magic trait promoting reproductive isolation in sticklebacks? Evol Ecol. 34(1):123–131.

Garnas JR. 2018. Rapid evolution of insects to global environmental change: conceptual issues and empirical gaps. Curr Opin Insect Sci. 29:93–101.

Georga I, Koumoundouros G. 2010. Thermally induced plasticity of body shape in adult zebrafish *Danio rerio* (Hamilton, 1822). J Morphol. 271(11):1319–1327.

Gilbert MJH, Harris LN, Malley BK, Schimnowski A, Moore J-S, Farrell AP. 2020. The thermal limits of cardiorespiratory performance in anadromous Arctic char (*Salvelinus alpinus*): a field-based investigation using a remote mobile laboratory. Conserv Physiol. 8(1):coaa036.

Gilbert SF, Epel D. 2015. Ecological developmental biology: the environmental regulation of development, health, and evolution. Oxford University Press.

Glazer AM, Cleves PA, Erickson PA, Lam AY, Miller CT. 2014. Parallel developmental genetic features underlie stickleback gill raker evolution. Evodevo. 5:1–16.

Greenwood AK, Peichel CL. 2015. Social regulation of gene expression in threespine sticklebacks. PLoS One. 10(9):e0137726.

Gunter HM, Fan S, Xiong F, Franchini P, Fruciano C, Meyer A. 2013. Shaping development through mechanical strain: the transcriptional basis of diet-induced phenotypic plasticity in a cichlid fish. Mol Ecol. 22(17):4516–4531.

Hairston NG Jr, Ellner SP, Geber MA, Yoshida T, Fox JA. 2005. Rapid evolution and the convergence of ecological and evolutionary time. Ecol Lett. 8(10):1114–1127.

Hansen AK, Byriel DB, Jensen MR, Steffensen JF, Svendsen MBS. 2017. Optimum temperature of a northern population of Arctic charr (*Salvelinus alpinus*) using heart rate Arrhenius breakpoint analysis. Polar Biol. 40(5):1063–1070.

Heath DD, Fox CW, Heath JW. 1999. Maternal effects on offspring size: Variation through early development of chinook salmon. Evolution. 53(5):1605–1611.

Heckwolf MJ, Meyer BS, Döring T, Eizaguirre C, Reusch TBH. 2018. Transgenerational plasticity and selection shape the adaptive potential of sticklebacks to salinity change. Evol Appl. 11(10):1873–1885.

Hegrenes S. 2001. Diet-induced phenotypic plasticity of feeding morphology in the orangespotted sunfish, *Lepomis humilis*. Ecol Freshw Fish. 10(1):35–42.

Heino, Kaitala. 1999. Evolution of resource allocation between growth and reproduction in animals with indeterminate growth. J Evol Biol. 12.

Hellmann JK, Bukhari SA, Deno J, Bell AM. 2020. Sex-specific plasticity across generations I: Maternal and paternal effects on sons and daughters. J Anim Ecol. 89(12):2788–2799.

Hellmann JK, Carlson ER, Bell AM. 2020. Sex-specific plasticity across generations II: Grandpaternal effects are lineage specific and sex specific. J Anim Ecol. 89(12):2800–2812.

Hendry AP, Peichel CL, Matthews B, Boughman JW, Nosil P. 2013. Stickleback research: the now and the next. Evol Ecol Res. 15(2):111–141.

Hendry AP, Taylor EB, McPhail JD. 2002. Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the Misty system. Evolution. 56(6):1199–1216.

Houle D, Govindaraju DR, Omholt S. 2010. Phenomics: the next challenge. Nat Rev Genet. 11(12):855–866.

Hynes HBN. 1950. The food of fresh-water sticklebacks (*Gasterosteus aculeatus* and *pygosteus pungitius*), with a review of methods used in studies of the food of fishes. J Anim Ecol. 19(1):36.

Islam MJ, Kunzmann A, Slater MJ. 2022. Responses of aquaculture fish to climate change-induced extreme temperatures: A review. J World Aquac Soc. 53(2):314–366.

Ives AR, Einarsson Á, Jansen VAA, Gardarsson A. 2008. High-amplitude fluctuations and alternative dynamical states of midges in Lake Myvatn. Nature. 452(7183):84–87.

Kamakura M. 2011. Royalactin induces queen differentiation in honeybees. Nature. 473(7348):478–483.

Kaplan HS, Robson AJ. 2009. We age because we grow. Proc Biol Sci. 276(1663):1837–1844.

Kim S-Y, Costa MM, Esteve-Codina A, Velando A. 2017. Transcriptional mechanisms underlying life-history responses to climate change in the three-spined stickleback. Evol Appl. 10(7):718–730.

Kimirei IA, Nagelkerken I, Trommelen M, Blankers P, van Hoytema N, Hoeijmakers D, Huijbers CM, Mgaya YD, Rypel AL. 2013. What Drives Ontogenetic Niche Shifts of Fishes in Coral Reef Ecosystems? Ecosystems. 16(5):783–796.

Kitano J, Kakioka R, Ishikawa A, Toyoda A, Kusakabe M. 2020. Differences in the contributions of sex linkage and androgen regulation to sex-biased gene expression in juvenile and adult sticklebacks. J Evol Biol. 33(8):1129–1138.

Krist M. 2011. Egg size and offspring quality: a meta-analysis in birds. Biol Rev Camb Philos Soc. 86(3):692–716.

Kristjánsson BK. 2001. Divergence of Icelandic threespine stickleback, *Gasterosteus aculeatus*, to two bottom types in lakes and recently formed lagoon. Noakes DLG, Skulason S, editors. MSc Thesis, The University of Guelph.

Kristjánsson BK, Skúlason S, Noakes DL. 2002. Morphological segregation of Icelandic threespine stickleback (*Gasterosteus aculeatus L.*). Biol J Linn Soc Lond. 76(2):247–257.

Kumawat B, Lalejini A, Acosta MM, Zaman L. 2025. Evolution takes multiple paths to evolvability when facing environmental change. Proc Natl Acad Sci USA. 122(1):e2413930121.

Lefébure R, Larsson S, Byström P. 2011. A temperature-dependent growth model for the three-spined stickleback *Gasterosteus aculeatus*. J Fish Biol. 79(7):1815–1827.

Levis NA, de la Serna Buzón S, Pfennig DW. 2015. An inducible offense: carnivore morph tadpoles induced by tadpole carnivory. Ecol Evol. 5(7):1405–1411.

Losos JB, Baum DA, Futuyma DJ, Hoekstra HE, Lenski RE, Moore AJ, Peichel CL, Schluter D, Whitlock MC. 2017. The Princeton Guide to Evolution. Princeton University Press.

Mariu A, Chatha AMM, Naz S, Khan MF, Safdar W, Ashraf I. 2023. Effect of temperature, pH, salinity and dissolved oxygen on fishes. J Zool Syst. 1(2):1–12.

Marshall JD, Uller T. 2007. When is a maternal effect adaptive? Oikos. 116(12):1957–1963.

Massey MD, Dalziel AC. 2023. Parental early life environments drive transgenerational plasticity of offspring metabolism in a freshwater fish (*Danio rerio*). Biol Lett. 19(10):20230266.

Matlosz S, Franzdóttir SR, Pálsson A, Jónsson ZO. 2024. DNA methylation reprogramming in teleosts. Evol Dev. 26(5):e12486.

Mehlis M, Rick IP, Bakker TCM. 2015. Dynamic resource allocation between pre- and postcopulatory episodes of sexual selection determines competitive fertilization success. Proc Biol Sci. 282(1817):20151279.

Mérigoux S, Ponton D. 1998. Body shape, diet and ontogenetic diet shifts in young fish of the Sinnamary River, French Guiana, South America. J Fish Biol. 52(3):556–569.

Merilä J, Hoffmann AA. 2016. Evolutionary impacts of climate change. In: Oxford Research Encyclopedia of Environmental Science.

Metzger DCH, Schulte PM. 2016. Maternal stress has divergent effects on gene expression patterns in the brains of male and female threespine stickleback. Proc Biol Sci. 283(1839).

Metzger DCH, Schulte PM. 2018. Similarities in temperature-dependent gene expression plasticity across timescales in threespine stickleback (*Gasterosteus aculeatus*). Mol Ecol. 27(10):2381–2396.

Meyer A. 1987. Phenotypic plasticity and heterochrony in *Cichlasoma managuense* (Pisces, Cichlidae) and their implications for speciation in cichlid fishes. Evolution. 41(6):1357–1369.

Millet A. 2013. Spatio-temporal variation of threespine stickleback (*Gasterosteus aculeatus*) in the dynamic Lake Mývatn, Iceland. Kristjánsson BK, Räsänen K, editors. PhD thesis, University of Iceland.

Millet A, Kristjánsson BK, Einarsson Á, Räsänen K. 2013. Spatial phenotypic and genetic structure of threespine stickleback (*Gasterosteus aculeatus*) in a heterogeneous natural system, Lake Mývatn, Iceland. Ecol Evol. 3(10):3219–3232.

Moczek AP, Sultan S, Foster S, Ledón-Rettig C, Dworkin I, Nijhout HF, Abouheif E, Pfennig DW. 2011. The role of developmental plasticity in evolutionary innovation. Proc Biol Sci. 278(1719):2705–2713.

Moosmann M, Greenway R, Brodersen J, Matthews B. 2023. Differential response of body size and head size to predator mediated resource limitation causes phenotypic variation among stickleback populations. Matthews B, Seehausen O, editors. PhD thesis, University of Bern.

Morris MRJ, Richard R, Leder EH, Barrett RDH, Aubin-Horth N, Rogers SM. 2014. Gene expression plasticity evolves in response to colonization of freshwater lakes in threespine stickleback. Mol Ecol. 23(13):3226–3240.

Mousseau TA, Fox CW. 1998a. The adaptive significance of maternal effects. Trends Ecol Evol. 13(10):403–407.

Mousseau TA, Fox CW. 1998b. Maternal effects as adaptations. Oxford University Press.

Ólafsdóttir GÁ, Snorrason SS, Ritchie MG. 2007. Postglacial intra-lacustrine divergence of Icelandic threespine stickleback morphs in three neovolcanic lakes. J Evol Biol. 20(5):1870–1881.

Ólafsson J. 1979. Physical Characteristics of Lake Mývatn and River Laxá. Oikos. 32(1/2):38–66.

Olsson J, Svanbäck R, Eklöv P. 2007. Effects of resource level and habitat type on behavioral and morphological plasticity in Eurasian perch. Oecologia. 152(1):48–56.

Panserat S, Hortopan GA, Plagnes-Juan E, Kolditz C, Lansard M, Skiba-Cassy S, Esquerré D, Geurden I, Médale F, Kaushik S, et al. 2009. Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. Aquaculture. 294(1):123–131.

Peichel CL, Marques DA. 2017. The genetic and molecular architecture of phenotypic diversity in sticklebacks. Philos Trans R Soc Lond B Biol Sci. 372(1713).

Petitgas P, Rijnsdorp AD, Dickey-Collas M, Engelhard GH, Peck MA, Pinnegar JK, Drinkwater K, Huret M, Nash RDM. 2013. Impacts of climate change on the complex life cycles of fish. Fish Oceanogr. 22(2):121–139.

Pfennig DW. 2021. Phenotypic Plasticity & Evolution: causes, consequences, controversies. Taylor & Francis (Evolutionary Cell Biology).

Pfennig DW, Wund MA, Snell-Rood EC, Cruickshank T, Schlichting CD, Moczek AP. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol Evol. 25(8):459–467.

Phillips JS, Einarsson Á, Strickland K, Ives AR, Kristjánsson BK, Räsänen K. 2023. Demographic Basis of Spatially Structured Fluctuations in a Threespine Stickleback Metapopulation. Am Nat. 201(3):E41–E55.

Poizat G, Rosecchi E, Crivelli AJ. 1999. Empirical evidence of a trade–off between reproductive effort and expectation of future reproduction in female three-spined sticklebacks. Proc R Soc Lond B Biol Sci. 266(1428):1543–1548.

Pörtner H-O, Berdal B, Blust R, Brix O, Colosimo A, De Wachter B, Giuliani A, Johansen T, Fischer T, Knust R, et al. 2001. Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). Cont Shelf Res. 21(18–19):1975–1997.

Post JR, Parkinson EA. 2001. Energy allocation strategy in young fish: Allometry and survival. Ecology. 82(4):1040.

Ramler D, Mitteroecker P, Shama LNS, Wegner KM, Ahnelt H. 2014. Nonlinear effects of temperature on body form and developmental canalization in the threespine stickleback. J Evol Biol. 27(3):497–507.

Ravinet M, Prodöhl PA, Harrod C. 2013. Parallel and nonparallel ecological, morphological and genetic divergence in lake-stream stickleback from a single catchment. J Evol Biol. 26(1):186–204.

Reid K, Bell MA, Veeramah KR. 2021. Threespine stickleback: A model system for evolutionary genomics. Annu Rev Genomics Hum Genet. 22(1):357–383.

Robinson B. 2000. Trade offs in habitat-specific foraging efficiency and the nascent adaptive divergence of sticklebacks in lakes. Behaviour. 137(7–8):865–888.

Salinas S, Munch SB. 2012. Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. Ecol Lett. 15(2):159–163.

Schlichting CD, Pigliucci M, Others. 1998. Phenotypic evolution: a reaction norm perspective. Sinauer Associates Incorporated.

Schluter D. 1993. Adaptive radiation in sticklebacks: Size, shape, and habitat use efficiency. Ecology. 74(3):699–709.

Schluter D. 2003. The Ecology of Adaptive Radiation. OUP Oxford.

Shama LNS. 2015. Bet hedging in a warming ocean: predictability of maternal environment shapes offspring size variation in marine sticklebacks. Glob Chang Biol. 21(12):4387–4400.

Shama LNS, Mark FC, Strobel A, Lokmer A, John U, Wegner KM. 2016. Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. Evol Appl. 9(9):1096–1111.

Shama LNS, Strobel A, Mark FC, Wegner KM. 2014. Transgenerational plasticity in marine sticklebacks: maternal effects mediate impacts of a warming ocean. Funct Ecol. 28(6):1482–1493.

Shama LNS, Wegner KM. 2014. Grandparental effects in marine sticklebacks: transgenerational plasticity across multiple generations. J Evol Biol. 27(11):2297–2307.

Singaraja RR, James ER, Crim J, Visscher H, Chatterjee A, Hayden MR. 2005. Alternate transcripts expressed in response to diet reflect tissue-specific regulation of ABCA1. J Lipid Res. 46(10):2061–2071.

Skúlason S, Parsons KJ, Svanbäck R, Räsänen K, Ferguson MM, Adams CE, Amundsen P-A, Bartels P, Bean CW, Boughman JW, et al. 2019. A way forward with eco evo devo: an extended theory of resource polymorphism with postglacial fishes as model systems. Biol Rev Camb Philos Soc. 94(5):1786–1808.

Snorradóttir RG. 2023. Relationships between phenotype, diet, and individual specialization of threespine stickleback. Kristjánsson BK, editor. MSc thesis, Hólar University.

Sogard SM. 1997. Size-Selective Mortality in the Juvenile Stage of Teleost Fishes: A Review. Bull Mar Sci. 60(3):1129–1157.

Sollid J, Nilsson GE. 2006. Plasticity of respiratory structures - adaptive remodeling of fish gills induced by ambient oxygen and temperature. Respir Physiol Neurobiol. 154(1–2):241–251.

Stearns SC. 1998. The evolution of life histories. Oxford University Press.

Steinberg CEW. 2018. Transgenerational effects – ‘your offspring will become what you eat.’ In: Aquatic Animal Nutrition: A Mechanistic Perspective from Individuals to Generations. Cham: Springer International Publishing. p. 333–430.

Steward RA, de Jong MA, Oostra V, Wheat CW. 2022. Alternative splicing in seasonal plasticity and the potential for adaptation to environmental change. Nat Commun. 13(1):755.

Strickland K, Matthews B, Jónsson ZO, Kristjánsson BK, Phillips JS, Einarsson Á, Räsänen K. 2024. Microevolutionary change in wild stickleback: Using integrative time-series data to infer responses to selection. Proc Natl Acad Sci USA. 121(37):e2410324121.

Strickland K, Räsänen K, Kristjánsson BK, Phillips JS, Einarsson Á, Snorradóttir RG, Bartrons M, Jónsson ZO. 2023. Genome-phenotype-environment associations identify signatures of selection in a panmictic population of threespine stickleback. Mol Ecol. 32(7):1708–1725.

Sultan S, Stearns S. 2005. Environmentally contingent variation: Phenotypic plasticity and norms of reaction. Variation. 303–332.

Tawfik AI, Tanaka S, De Loof A, Schoofs L, Baggerman G, Waelkens E, Derua R, Milner Y, Yerushalmi Y, Pener MP. 1999. Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant. Proc Natl Acad Sci USA. 96(12):7083–7087.

Thunell V, Gårdmark A, Huss M, Vindenes Y. 2023. Optimal energy allocation trade-off driven by size-dependent physiological and demographic responses to warming. Ecology. 104(4):e3967.

West-Eberhard MJ. 1989. Phenotypic Plasticity and the Origins of Diversity. Annu Rev Ecol Syst. 20(1):249–278.

West-Eberhard MJ. 2003. Developmental Plasticity and Evolution. Oxford University Press.

Whitehead A, Crawford DL. 2006a. Variation within and among species in gene expression: raw material for evolution. Mol Ecol. 15(5):1197–1211.

Whitehead A, Crawford DL. 2006b. Neutral and adaptive variation in gene expression. Proc Natl Acad Sci USA. 103(14):5425–5430.

Wimberger PH. 1991. Plasticity of jaw and skull morphology in the neotropical cichlids *Geophagus brasiliensis* and *G. Steindachneri*. Evolution. 45(7):1545–1563.

Wimberger PH. 1992. Plasticity of fish body shape. The effects of diet, development, family and age in two species of *Geophagus* (Pisces: Cichlidae). Biol J Linn Soc Lond. 45(3):197–218.

Wootton RJ. 2012. Ecology of Teleost Fishes. Springer Science & Business Media.

Wund MA, Baker JA, Clancy B, Golub JL, Foster SA. 2008. A Test of the "Flexible Stem" Model of Evolution: Ancestral Plasticity, Genetic Accommodation, and Morphological Divergence in the Threespine Stickleback Radiation. Am Nat. 172(4):449–462.

Zhu K, Liu M, Fu Z, Zhou Z, Kong Y, Liang H, Lin Z, Luo J, Zheng H, Wan P, et al. 2017. Plant microRNAs in larval food regulate honeybee caste development. PLoS Genet. 13(8):e1006946.

2 Juvenile feeding ecology in threespine stickleback (*Gasterosteus aculeatus*)

Alessandra Schnider [1,2], Franka Hemme [1,3], Etienne de la Burgade [1,4], Bjarni Kristófer Kristjánsson[1]

[1] Department of Aquaculture and Fish Biology, Hólar University, Sauðárkrókur, Iceland
[2] Faculty of Life and Environmental Sciences, University of Iceland, Reykjavík, Iceland
[3] Faculty of Geosciences, University of Bremen, Bremen, Germany
[4] Ecole d'Ingénieur de PURPAN, Toulouse, France

2.1 Abstract

Threespine stickleback (*Gasterosteus aculeatus*) are known for their rapid adaptation to a wide range of environments. However, despite having served as a model species for ecology, evolution, and developmental biology, little is known about the ontological dietary changes of juvenile individuals in the wild. This has created a gap in our knowledge, given that the organismal phenotype is largely moulded during the juvenile phase, and natural selection during this life stage is often strong. Studying juvenile threespine stickleback from lake Mývatn offers an opportunity for a detailed study of the organism-environment relationship, because the lake is highly heterogenous in terms of habitat and resource availability, and the adult stickleback population is divergent in feeding morphology among contrasting habitats. Here, we tested whether diet, based on gut content analysis, and trophic morphology have diverged between juveniles from different habitats and if diet composition covaries with morphology and body size. Overall, we saw clear differences in diet composition among distinct habitats within the lake, and morphological divergence in head morphology (e.g. gape size) among contrasting habitats. Juveniles tended to either have many small Cladocera or Chironomidae larvae in their diet; smaller individuals tended to have more Cladocera, and bigger individuals consumed more Chironomidae larvae. Such an ontogenetic diet shift is in agreement with laboratory and other field studies on juvenile stickleback. However, this is one of the first studies, to investigate the association between juvenile diet and head morphology in wild freshwater stickleback. Thus, the study delivers unique insight into the development of trophic morphology, as well as diet-mediated diversification in threespine stickleback.

2.2 Introduction

The capacity of an individual to utilize its environment is crucial to its survival, reproduction and ultimately fitness. Traditionally, a well-adapted trait is interpreted as a highly specialized phenotype, shaped by a specific selection pressure, that maximises an individual's fitness in a specific environmental context (Schluter 1995; Ingram et al. 2012). However, environments and, consequently, resources are often not predictable and therefore a capacity to cope with highly variable environments in a plastic way may be favoured (West-Eberhard 1989; Nonaka et al. 2015). Furthermore, environments and selection pressures might vary drastically throughout development, and individuals might exploit different ecological niches across different developmental stages. Such ontogenetic niche shifts are reported across many taxa and can be driven by various factors such as predation pressure, intra- and interspecific competition, and individual differences in body size, condition and phenotype (Ingram et al. 2012; Kimirei et al. 2013; Rudolf 2020). However, despite many prominent examples of ontogenetic niche shift, for instance, in anadromous fish species, our knowledge on which traits underlie niche variation is limited (Rudolf 2020).

Ontogenetic shifts in diet are often marked by morphological changes through development. Changes in morphology can be as drastic as in flatfish that undergo metamorphosis, during which the eye migrates to the opposite side of the head (Gisbert et al. 2002). Often more subtle changes are found in allometry, where body proportions shift during development and/or growth, such as often observed in the relative growth of head and body size (Osse et al. 1997). However, in addition to allometric changes, many species grow through many orders of magnitude and thus show great size variation in their lifetime. This size variation can have consequences for predation avoidance, feeding, and competition for resources within and among species (Amundsen et al. 2003; Kimirei et al. 2013).

Competition for resources has been extensively studied, and many examples exist where a dietary niche shift has reduced competition. Indeed, diet-induced phenotypic divergence is common for many fish species and often manifests as resource polymorphism (Seehausen and Wagner 2014; Skúlason et al. 2019). For example, differentiation in diet between benthic and limnetic foraging habitats has been well established in a range of taxa such as cichlid, white fish (*Coregonus ssp.*), charr (*Salvelinus spp.*) and stickleback (*Gasterosteus spp.*). Fish feeding on a benthic diet tend to have a wider jaw with fewer and shorter gill rakers, and a sub-terminal mouth orientation, whereas individuals feeding on a limnetic diet have a narrower jaw with more numerous and longer gill rakers, and a more terminal mouth (Day et al. 1994; Cresko et al. 2007; Crichigno et al. 2014). The diet composition of individuals not only gives direct evidence of what an individual consumes but also gives insight into foraging behaviour, which allows interpretations of its niche use (Ingram et al. 2012; Bolnick and Ballare 2020). However, studies in the wild have focused almost exclusively on adult phenotypes. Individuals are generally most vulnerable in early life stages and thus, changes in environment could be especially taxing for juveniles, further strengthening selection pressures acting during these different and often diverse life stages.

Threespine stickleback (*Gasterosteus aculeatus*) have been extensively studied and diverging morphs or ecotypes along axis's of ecological divergent resource use have been repeatedly described (Robinson 2000; Kristjánsson 2001; Hendry et al. 2002). Stickleback has been called a (super)model organism for the study of phenotypic variation and

evolution (Hendry et al. 2013). However, studies have been heavily focused on the fully formed adult phenotype (Day and McPhail 1996; Garduno-Paz et al. 2010). Importantly, the phenotype develops continuously throughout the juvenile life stage and is likely extensively impacted by the juvenile ecology, which can be quite different from the adult´s (Jonsson and Jonsson 2014). Consequently, the adult phenotype might not always be fully explained by the current environment, meaning we need to study juvenile individual morphology and diet to better understand the phenotypic expression of adults.

However, only few studies have examined juvenile stickleback diet in the wild (Delbeek and Williams 1988; Hangelin and Vuorinen 1988; Demchuk et al. 2015), and even fewer in freshwater habitats (Hynes 1950). These studies reported that diet was mainly governed by prey availability and mouth size, with fish with wider mouths feeding on larger prey items. A study in the White Sea found a dietary shift from small Crustaceans onto a Chironomidae with increasing body length, indicating an ontogenetic shift in diet (Demchuk et al. 2015). Moreover, most studies investigating head shape in relation to diet in stickleback have overwhelmingly been focused on adult individuals. There is, thus, a clear gap in our knowledge of the feeding ecology of wild juvenile stickleback. This is important to study, since threespine stickleback are an integral part of freshwater systems across the entire northern hemisphere. Thus, understanding how juvenile individuals interact with their environment is central to identify potential limitations to their development and recruitment. This will allow us to better understand phenotypic variation in adults and how stickleback ecology develops throughout their lifetime.

Threespine stickleback from lake Mývatn, northern Iceland, are a well-suited study system to investigate ontogenetic niche shift, as this single panmictic population is found across strong environmental gradients (e.g. substrate and temperature) across the lake (Millet et al. 2013; Strickland et al. 2023). They further experience great seasonal fluctuations prey availability (Ives et al. 2008; Bartrons et al. 2015; Phillips et al. 2023). Especially impactful is the fluctuation in Chironomidae densities within and across years, which appears to be the main driver of fluctuations across all trophic levels of the Mývatn system (Phillips et al. 2019). Indeed, the trophic morphology of stickleback, such as gill raker structure, as well as diet composition has been found to be associated with contrasting habitats, and difference in prey availability within and across seasons (Millet 2013; Strickland et al. 2023).

The presented study describes the juvenile feeding ecology of wild threespine stickleback from lake Mývatn. Specifically, we aim to understand how diet composition varies across habitats and with body size. Furthermore, in light of the aforementioned knowledge gap, we aim to test the association between diet and head shape. By studying wild individuals, we can improve our understanding of the selection pressures acting on the developing phenotype and better contextualize environmentally driven phenotypic divergence of adult populations.

2.3 Methods

2.3.1 Study system

Lake Mývatn (65°36'N, 17°00'W; 278m a.s.l.) in north-eastern Iceland is a shallow eutrophic lake, formed around 2'300 years ago following a volcanic eruption (Fig. 2.1) (Einarsson et al. 2004). Its 37km^2 area is divided into two main basins connected by two narrow channels: the smaller, spatially more heterogeneous and deeper (1-6m depth) north basin and the more homogenous and shallower (2-3.2m depth) south basin (Einarsson and Örnólfsdóttir 2004; Phillips et al. 2023). The lake is fed by cold water springs (~5°C) in the south-east and geothermal hot springs (~23°C) in the north-east. These springs form the "Warm" habitat with average year-round water temperature of 20-23°C, and a cold habitat which is generally uninhabited by stickleback. The rest of the lake's water temperature fluctuates with seasonal air temperature with average summer temperatures of 13-23°C (Ólafsson 1979; Millet et al. 2013). The lake further varies in productivity, abundance and community structure of invertebrate (Einarsson and Örnólfsdóttir 2004; Ives et al. 2008; Bartrons et al. 2015).

Based on temperature, depth, substrate and vegetation, five habitats populated by stickleback had previously been described as warm, mined, rocky shore, cladophorales, and pondweed (Millet et al. 2013; Strickland et al. 2023). The invertebrate community of the lake is dominated by Chironomidae and Cladocera, which constitute the primary food source of adult stickleback (Millet et al. 2013; Bartrons et al. 2015; Snorradóttir 2023). For the current study on morphology and diet of juvenile threespine stickleback, individuals across the five habitats defined within Lake Mývatn were studied (Millet et al. 2013) (Fig. 2.1).

2.3.2 Sampling and data collection

The stickleback breeding season in Lake Mývatn spans from early June to early August. Therefore, we sampled in late August 2021 to capture a wide range of juvenile ages and sizes. We sampled a total of 151 fish from five different sites, representing five habitat types, "Cladophorales" (station "23") N = 36, "Pondweed" (station "135") N = 32, "Warm" (station "HS") N = 31, "Mined" (station "124") N = 12, "Shore" (station "GR") N = 40. We used unbaited minnow traps (laid for 2-12h) covered in transparent plastic to catch juveniles of all sizes. Fish were subsequently fixed in 95% EtOH for processing. Each fish was weighed, and total length measured before dissecting out the stomach and intestine. All fish smaller than 35mm were included in this study. We based this cut-off on previous work reporting that bone morphology, especially armour plates, at that length is fully formed and reflects the adult morphology (Bell 1981).

Dietary items found in the stomach were identified and counted under a dissecting microscope to the lowest possible taxa: small Cladocera (*Alona* spp., *Chydorus sphaericus*, *Alonella nana*, *Graptoleberis testudinaria*, *Acroperus harpae*), big Cladocera (*Daphnia longispina*, *Macrothrix hirsuticornix*, *Eurycercus lamellatus*, *Simocephalus vetulus*), Chironomidae larvae, adult flies and Other diet items (e.g. snails, mulluscs and terrestrial insects). The grouping within Cladocera was made based on physical size as well as which species are predominantly associated with a benthic or epibenthic environment. Likewise, we separated Chironomidae larvae and adult flies because the larvae are associated with

the benthos, whereas adult flies are found close to the water surface. Proportions of the major food groups were calculated for each individual based on the count data.

To visualise bone morphology, fish were stained, post dissection, using an Alizarin red staining protocol. Individuals were rinsed in a 1% KOH solution for five minutes, then bleached for four hours in solution of 0.88% H_2O_2 and 0.71% KOH until the fish's eyes were brown and the body white. After rinsing the fish in a 1% KOH solution, they were stained with an Alizarin red solution (300mg/L in 1% KOH) for three to four hours until the bones were coloured purple. Subsequently the fish were rinsed in a 1% KOH solution for 15 minutes and rinsed under light water flow over night. The following morning fish were returned into a 70% EtOH solution for storage. Finally, the left side of all individuals was photographed, with a scale for calibration, to characterise head morphology. To this end a *Canon EOS 750D* camera with a *Canon 50mm* macro lens was mounted on a white light box to minimize shadows.

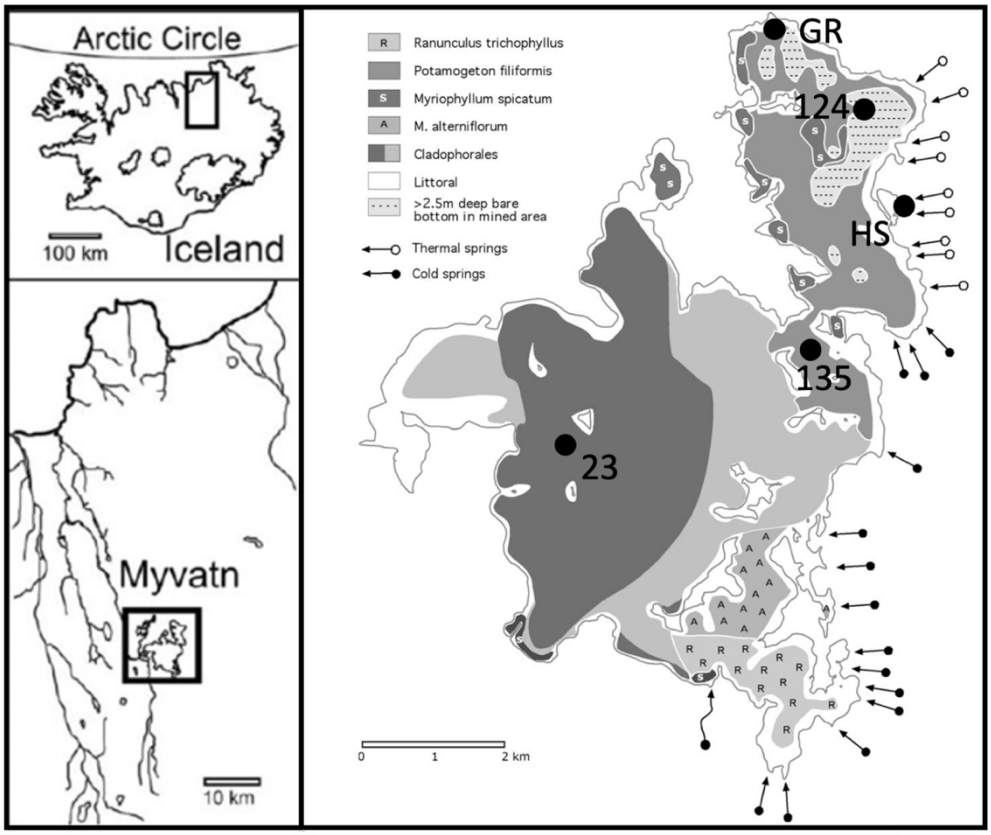

Figure 2.1 *Map of Lake Mývatn, NE Iceland, depicting the dominant benthic macrophytes (in late 2000) and the main spring water inflows. The two shades of grey used to describe the Cladophorales habitats reflect density (lighter: less dense, darker: denser). Black circles mark the five sampling sites of threespine stickleback representing the five habitats: "Shore" (labelled "GR"), "Mined" (labelled "124), "Warm" (labelled "HS"), "Pondweed" ("135") and "Cladophorales" (labelled "23"). Edited from Einarsson et al. (2004).*

2.3.3 Statistical analyses

Head morphology analyses

The images of stained fish were converted into *tps* files using *tpsUtil*. To capture cranial morphology, we placed 14 fixed landmarks and 8 sliding semi-landmarks in *tpsDig2* v.2.32 (McGee et al. 2013) (Fig. 2.2). We performed a generalized Procrustes analysis using the "gpagen" function from the *geomorph* package in *RStudio* (Posit team 2023) to rotate, scale and align landmarks. This produced 44 shape variables (X and Y coordinates for 22 landmarks) which are used for all following analyses. We tested for allometric changes performing a regression for Procrustes shape variables (Procrustes ANOVA) with log centroid size and habitat as a predictor using the "*procD.lm*" function from the *geomorph* package. We found common allometric changes with different means among habitats. We therefore corrected for allometric changes by adding the residuals from the Procrustes ANOVA, with log centroid size as predictor, to the mean shape generated with the "mshape" function from the *geomorph* package. Once corrected for allometric changes, we tested for morphological differences among habitats using a Procrustes ANOVA. We did also run a model with both size and origin, which did not change the results in regards to effect of origin on shape in a meaningful manner. Additionally, we then ran a principal component analysis for the uncorrected shape data using the "gm.prcomp" function from the *geopmorph* package to visualize the shape differences among the different habitats. To further visualize shape differences along the PC axes we estimated extreme head shapes based on the PC values from each axis using the "shape.predictor" and "plotRefToTarget" functions in the *geomorph* package.

Associations between diet composition and head morphology

We tested for associations between head morphology and diet composition with a two-block partial least squares analysis for Procrustes shape variables within the *geomorph* package ("two.b.pls" function). The function returns a correlation coefficient of projected values on the first singular vectors between the left (shape coordinates) and right block (diet proportion). It also returns a p-value from the resampling procedure, as well as the singular vectors of the right and left block.

For diet analyses we used the proportions for each diet group per fish in PERMANOVAs to test for the effect of habitat and total body length on diet composition. As body length differed between habitats, we ran two independent models for these two explanatory variables. To test for associations between individual diet groups and total body length we used an independent generalized linear model for each major diet group (small Cladocera, big Cladocera, Chironomidae larvae, adult Chironomidae, total various). Finally, we performed non-metric multidimensional scaling (NMDS; k = 4, stress = 0.028, non-metric fit, $R^2 = 0.999$ and linear fit, $R^2 = 0.996$) using the "*metaMDS*" function from the "*vegan*" package. It is a robust unconstrained ordination method which standardises and scales results, facilitating the visualisation of multidimensional data.

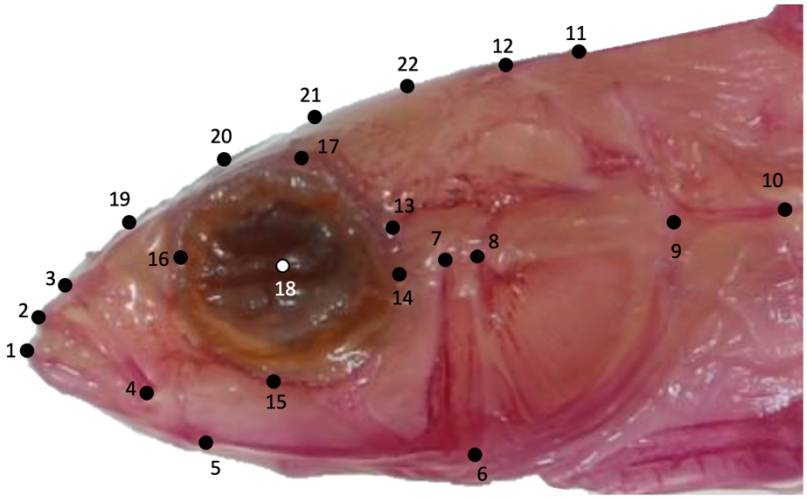

Figure 2.2 *Lateral photograph of the cranial section of a stained threespine stickleback juvenile of Lake Mývatn illustrating the position of the 22 landmarks used in this study. Fixed landmarks were: 1-3: Anterior lower, middle and upper lip, 4: Posterior end of the mouth, 5: Posterior end of the mandible, 6: lowermost point of the pre-operculum, 7: Uppermost point of the pre-operculum, 8: Opercular joint, 9: Anterior part of the cleithrum centred by landmark 10, 10: most posterior end of the cleithrum, 11: supraoccipital notch lateral to dorsal midline, 12: Posterior dorsal edge of cranium, 13: junction between the post-orbital and the supraoccipital bone, 18: Centre of the eye. Sliding landmarks were: 14: The posterior-most point of the orbital circumference, 15: The lowermost point of the orbital circumference, 16: The anterior-most point of the orbital circumference, 17: The uppermost point of the orbital circumference and 19-22: Landmarks are placed in equidistance between landmarks 3 and 12 to capture neurocranial shape.* (McGee et al. 2013; Snorradóttir 2023).

2.4 Results

2.4.1 Body length and head morphology

Body length ranged from 15.6mm to 34.8mm with a mean body length of 24.95 ± 4.479mm. Fish from Shore (mean = 27.82 ± 3.376mm) and Mined (mean = 27.77 ± 5.785mm) were the largest among the data set, followed by fish from Pondweed (mean = 25.62 ± 4.000mm) and Warm (mean = 24.15 ± 3.501mm). Fish from Cladophorales were clearly the smallest (mean = 20.91 ± 2.857mm).

The Procrustes ANOVA on the shape coordinates showed that head shape changed substantially with body length (R^2 = 0.068, Df = 1, F = 10.90, Z = 5.01, p < 0.001) with smaller individuals having shorter and more rounded snouts. The allometric changes were common among sites, only with means differing, as no interaction between site and body length was detected. After correcting for these allometric changes, we observed differences in head shape among habitats (R^2 = 0.071, Df = 4, F = 2.77, Z = 3.86, p < 0.001). PC1

explained 31% of variance and appears to separate the two lake basins whereby the three north basin habitats ("Mined", "Shore", "Warm") scored positively, and the two south basin habitats ("Cladophorales", "Pondweed") negatively (Fig 2.3). PC2 explained 16% of variance and further separates the habitats except for "Warm" and "Mined" habitat that clustered closely together on this axis (Fig 2.3). PC3 only explained 9% of variation and showed no association with habitat, size or diet composition. Overall, the largest morphological changes were observed in the anterior part of the head. Specifically, the nasal ridge became increasingly rounded along the PC1 axis, rendering the whole snout shape slightly longer and more rounded towards the PC1 maximum (Fig 2.3). Along the PC2 axis, the distance between the tip of lower lip (landmark 1) and the posterior end of the mouth (landmark 4) was progressively more elongated with increasing PC2 values. Consequently, the mouth gape was wider at lower PC2 values (Fig 2.3).

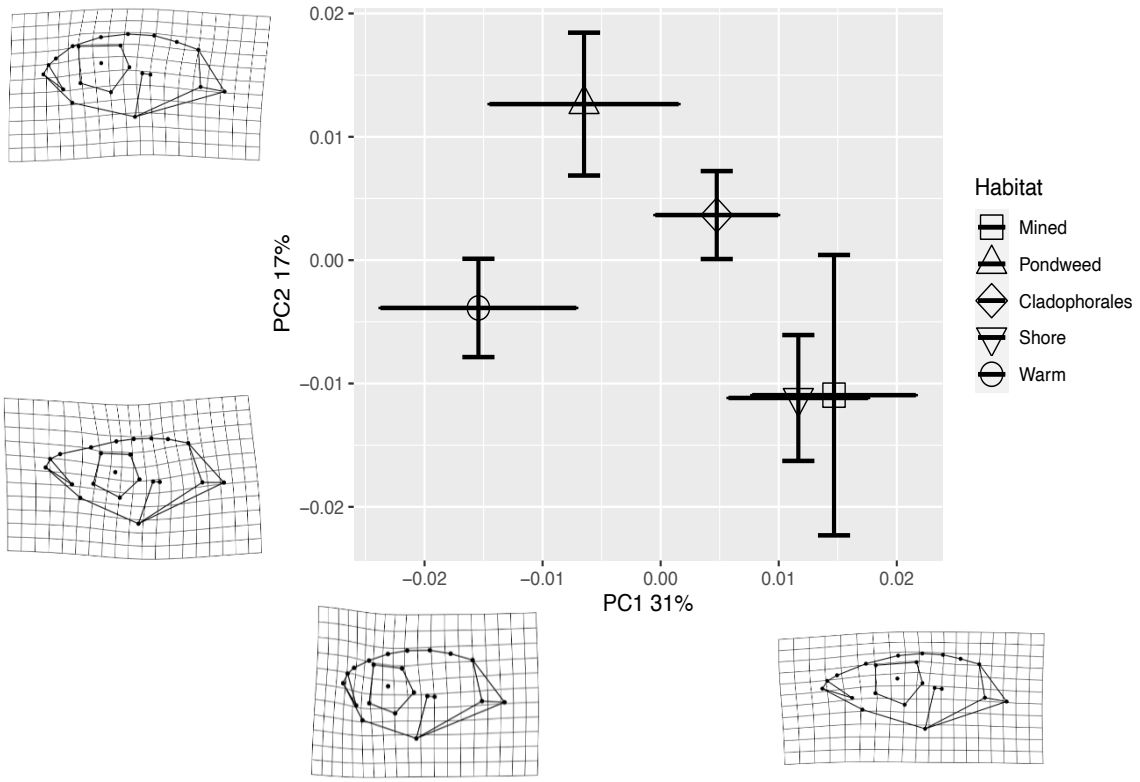

Figure 2.3 *Principal Component Analysis (PCA) of head shape of juvenile threespine stickleback. The cranial morphology for each individual was captured by placing 22 geometric morphometric landmarks (see Fig. 2.2). The landmarks were converted into 44 scaled and aligned shape variables (X and Y coordinates for 22 landmarks) using a Generalized Procrustes Analysis (GPA). These shape variables were then used to run a PCA (see methods section for further details). Each point represents the mean shape for the respective habitat and lines represent the standard deviation in each habitat (see legend). The deformation grids were plotted with a 1 magnification. The deformation grids represent the shape at the extreme end of each PC axis.*

2.4.2 Diet composition among habitats

Juvenile stickleback stomach content in lake Mývatn mainly consisted of Cladocera (small 48.0%, large 9.2%) and Chironomidae (larvae 23.6%, adult 11.8%). There was however also a wide variety of other diet items, such as snails, bivalves, mites, ostracods, copepods, rotifers, stickleback eggs, tubifex and terrestrial insects that together made up 7.4% of the juvenile stomach content. Further, in 24% of the stomachs a substantial number of diatoms and algae were present.

Overall, diet composition varied among habitats (R^2 = 0.31, Df = 4, F = 16.34, p = 0.001), but also changed slightly with body length (R^2 = 0.04, Df = 1, F = 6.87, p = 0.003). Specifically, the proportion of small Cladocera decreased with increasing body size (R^2 = 0.06, t = -3.44, p < 0.001), whereas the proportion of adult Chironomidae increased with increasing body size (R^2 = 0.11, t = 3.82, p < 0.001). However, as mentioned above, body size differed among habitats. From the NMDS it is evident that there is a variation in diet breadth, indicated by the size of the ellipsis representing the mean diet per habitat. Further,

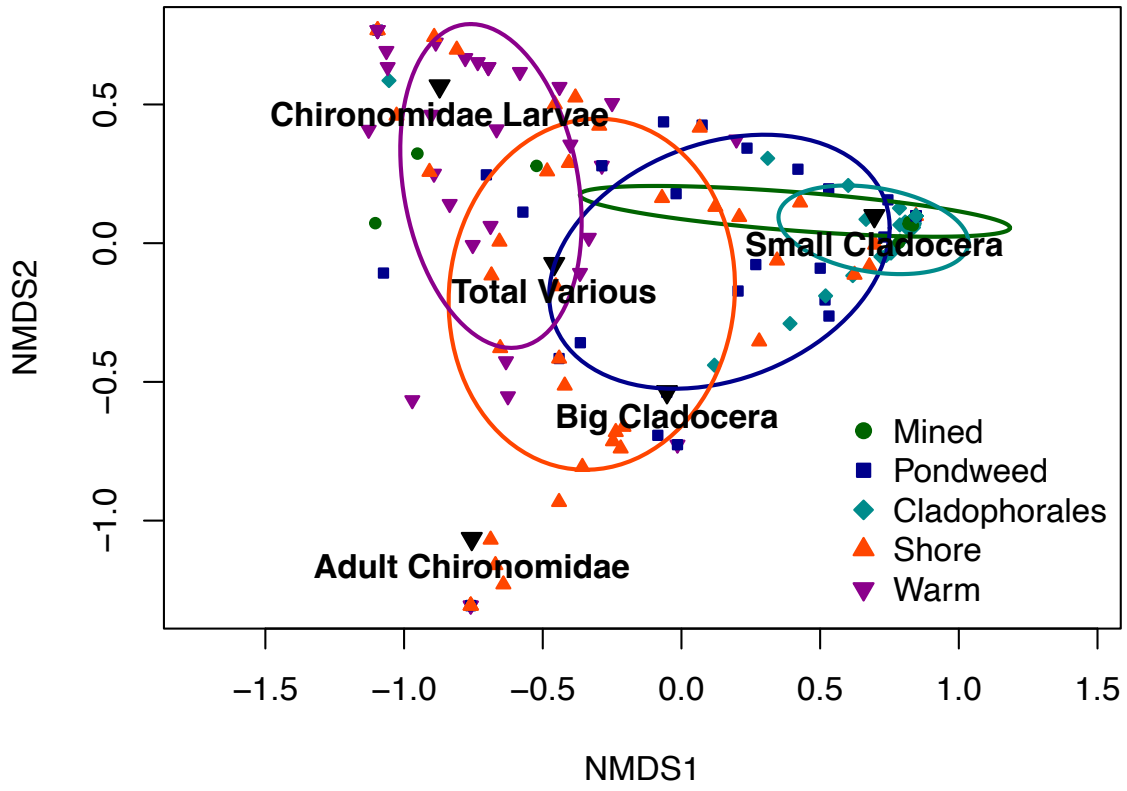

Figure 2.4 *Non-metric multidimensional scaling (NMDS) ordination plot illustrating diversity among diet composition of juvenile threespine stickleback across contrasting habitats (indicated in the inset). A Bray-Curtis distance similarity matrix was calculated based on diet composition of 151 individuals. Each point represents an individual in the colour of the respective habitat it was caught. The shorter the distance between two individuals, the more alike they are. Ellipses represent the standard deviation of the weighted mean of NMDS scores for each habitat. Black triangles indicate the major diet groups (see methods for further description of the diet groups). The NMDS was generated with k = 4 (stress = 0.028, non-metric fit, R^2 = 0.999 and linear fit, R^2 = 0.996).*

there is substantial overlap between some habitats. Indeed, individuals from habitats further away from the shore ("Mined", "Pondweed", "Cladophorales") cluster around small Cladocera associated with positive NMDS1 values, whereas individuals from habitats at the shore ("Shore", "Warm") in contrast cluster closer to Chironomidae, associated with negative NMDS1 values (Fig 2.4).

2.4.3 Association between diet composition and head morphology

The two-block partial least squares analysis for Procrustes shape variables showed a moderate association between the head morphology (left block) and diet composition (correlation coefficient $r = 0.045$, $p = 0.001$, effect size $Z = 3.5$). From the first vector on the right side (diet proportion) it is evident that small Cladocera have a positive association with morphology, whereas Chironomidae larvae have a negative association with the first morphology vector (Table 2.1). Plotting the extreme head shapes, uncorrected for size, onto the first morphology vector it is apparent that a head shape indicative of earlier developmental stages (shorter and rounder snout and larger eye) is associated with a diet consisting of small Cladocera, whereas a more a Chironomidae based diet is associated with a more pointed and longer snout and a relatively smaller eye indicative of a more developed head shape (Fig. 2.5, Supplementary Fig. A.1).

Table 2.1 *The table lists the first four singular vectors of the right block (diet composition) resulting from a two-block PLS analysis of Procrustes shape variables, capturing head shape (Supplementary Fig. A.1), and diet composition of 151 juvenile threespine stickleback caught in five contrasting habitats within lake Mývatn. Positive values represent positive associations between a major diet group and head shape values, whereas negative values represent negative associations. Percentages indicate the variance explained.*

Diet Group	**Vector 1** 75%	**Vector 2** 14%	**Vector 3** 8%	**Vector 4** 3%
Small Cladocera	0.76	-0.46	-0.09	0.05
Big Cladocera	0.04	0.61	-0.63	-0.17
Adult Chironomidae	-0.03	0.18	0.65	-0.58
Chironomidae larvae	-0.63	-0.57	-0.26	-0.09
Various	-0.14	0.23	0.32	0.79

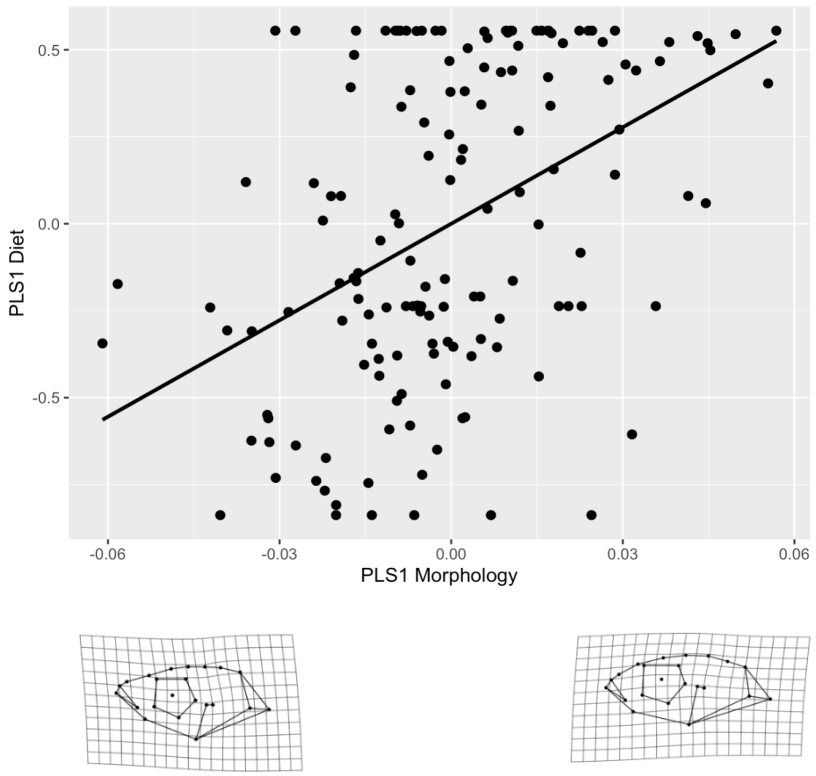

Figure 2.5 *Quantitative relationship between head morphology and diet composition of juvenile threespine stickleback caught in five contrasting habitats within lake Mývatn. The two-block partial least squares analysis for Procrustes shape variables shows a moderate association between the head morphology and diet (correlation coefficient r = 0.045, P = 0.001, effect size Z = 3.5). The best fit line illustrates the statistical association between morphology (left block) and diet composition (right block). A positive PLS1 Diet value is associated with a higher proportion of Cladocera in the diet. Deformation grids represent the shape at the extreme ends of PLS1 axis. The deformation grids were plotted with a 1 magnification. A more shorter and rounded head shape with a larger eye thus, is associated with a Cladocera based diet, whereas a more elongated head shape and smaller eye is associated with a Chironomidae based diet.*

2.5 Discussion

The overwhelming majority of studies on phenotypic divergence in response to trophic ecology have focused on describing ecological adaptation to contrasting diets in adults. In fish, especially the benthic-limnetic contrast has been explored, including the connection between morphology and diet (Day et al. 1994; Cresko et al. 2007; Berner et al. 2008). However, in a shallow lake like Mývatn such a divergence is not to be expected, rather previous studies on this populations found phenotypic divergence along other environmental gradients (Millet et al. 2013; Strickland et al. 2024). Multiple studies have tested how ontogenetic niche shift can drive habitat use in varying developmental stages and can contribute to phenotypic divergence in adults (Amundsen et al. 2003; Hjelm et al. 2003; Ingram et al. 2012). Here we study morphological and dietary variation in juvenile stickleback across multiple habitat types to test for an ontogenetic dietary shift. This could shed light on phenotypic divergence observed in adult threespine stickleback in lake Mývatn, in the absence of any clear genetic divergence (Strickland et al. 2023).

2.5.1 Head morphology

We found substantial allometric changes in head morphology, with major changes concentrating on the shape and length of the snout (space between the eye and anterior tip of the head). Specifically, small individuals had a shorter and more rounded snout, whereas larger individuals had a more elongated and protruding snout. These patterns are commonly observed in developmental patterns across fish species (Skúlason et al. 1989; Gisbert et al. 2002), confirming that we likely have fish that vary in development in our data set. After correction for allometric changes, there were clear differences among habitats in juvenile morphology, with habitat explaining around 7% of morphological variation. Most of this variation seemed to be related to differences between fish in the north and south basin– whereby north basin juveniles had a longer and more rounded snout than south basin juveniles of the same size. The second PC axis shows an increase in mouth gape which has a clear association with diet, whereby elongated snouts and smaller mouth gapes are associated with a more limnetic diet, and shorter, more rounded snouts and bigger mouth gapes are associated with a predominantly benthic diet (Wund et al. 2008; Matthews et al. 2010).

2.5.2 Juvenile diet

Our results show that juvenile stickleback from Mývatn predominantly feed on Cladocera and Chironomidae, as seen in several studies of adults from this lake (Kristjánsson et al. 2002; Millet et al. 2013; Snorradóttir 2023). However, we also found a wide variety of other prey items such as Rotifers, Copepods, Ostracoda, Mollusca, Bivalves, mites and terrestrial insects. The best predictor of diet composition was habitat type, which is consistent with results obtained from adults (Millet et al. 2013; Snorradóttir 2023; Strickland et al. 2023), particularly for fish caught close to the shore compared to further out in the lake (Fig 2.4). Given the high spatial and temporal variation in zooplankton and Chironomidae presence in lake Mývatn it is not surprising that diet composition is largely driven by local food availability (Einarsson and Örnólfsdóttir 2004; Ives et al. 2008; Bartrons et al. 2015; Phillips et al. 2023).

We found clear evidence for an ontogenetic diet from small Cladocera to adult Chironomidae as individuals increased in body size. This transition not only reflects an increase in prey size but likely indicates a change in habitat use. Most small Cladocera in our data belong to *Alona* spp. which are found close to the benthos or associated with benthic vegetation (epibenthos), whereas adult Chironomidae are found at the water surface during emergence. In threespine stickleback early survival is tightly linked to body size (Reimchen 1990), thus, smaller individuals tend to spend more time close to the benthos where they are more sheltered from predators. Predation pressure is a known driver of niche change, such as in coral reef fish, where juveniles inhabit less profitable habitats, compared to adults, as a trade-off for reduced predation pressure (Kimirei et al. 2013).

We also found that the variety of diet items increased with increasing body size. Consequently, the largest variation in diet composition was observed in the north basin Shore habitat where fish are on average the biggest. In contrast, the lowest diet variation was observed in the south basin Cladophorales habitat where the fish are on average the smallest. Laboratory experiments have demonstrated that threespine stickleback mouth width limits the prey size they can successfully hunt and consume (Gill and Hart 1994).

Consequently, the smaller individuals found in the Cladophorales habitat may be physically incapable to handle larger prey such as adult Chironomidae, big Cladocera and terrestrial insects. Thus, with increasing fish size and therefore mouth gape, the prey options increase as well. However, all habitats overlap in their size distribution, except for the Cladophorales and Shore habitat which limits the inferences on diet composition based on body length.

Our findings about ontogenetic diet shifts are well aligned with previous studies on juvenile stickleback diet from other systems. In fact, from saltwater, brackish to freshwater systems, there is a general dietary ontogeny emerging, where small juveniles predominantly feed on small crustaceans and with increasing body size shift towards Chironomidae (Delbeek and Williams 1988; Hangelin and Vuorinen 1988; Demchuk et al. 2015). This could indicate that small crustaceans are a less favourable prey compared to Chironomidae and are only consumed in the early stages of development, when individuals are physically incapable to feed on larger prey. However, the dietary repertoire is not purely determined by what individuals are physically able to consume but also prey availability, shaping the habitat and size-dependent pattern we observe in this study.

2.5.3 Association between diet and head morphology

We found a slight association between diet and head shape. This association is particularly evident in the second PC2 axis which relates to increasing proportion of small Cladocera in an individual's diet and a narrower mouth on one end versus a Chironomidae dominated diet and a wider mouth on the other end. Additionally, the first PLS vector shows individuals with a more rounded head shape, which is associated with earlier developmental stages, feeding on Cladocera, whereas individuals with a more developed head shape fed on Chironomidae larvae. Together, diet and morphology make a strong case for an ontogenetic niche shift driven by body size. Indeed, early survival in stickleback is mainly driven by size, thus, growing faster may increase survival by decreasing size-dependent predation (Reimchen 1990). Therefore, it is possible that head shape in early development is predominantly shaped by optimizing feeding to maximize growth. Whereas once a critical size has been reached other selection pressures might become more important and act in a different direction than diet. However, in our study we could not distinguish if individuals developed a certain head shape in response to the diet consumed or if fish with a certain head preferred particular diet items. From laboratory studies on stickleback it is known that diet can produce phenotypic changes in head and overall body morphology and that a specialised trophic morphology can increase feeding efficiency (Garduno-Paz et al. 2010; Moosmann et al. 2022). Thus, the development of feeding ecology is clearly an interaction between the phenotype and the environment rather than a unilateral effect we can observe in laboratory experiments with simplified environments (Skúlason et al. 2019).

Interestingly, a recent study on adult stickleback from Mývatn found divergence in head shape and diet among habitats, but no co-variation between those two traits (Snorradóttir 2023). However, gill raker structure of adult stickleback has been found to differ among habitats and co-vary with diet composition (Kristjánsson et al. 2002; Millet et al. 2013; Snorradóttir 2023; Strickland et al. 2023). This could mean that head shape is plastic in response to diet early in development, whereas other factors later in development counteract this initial divergent pattern found in juvenile head morphology. Indeed (Millet

2013) observed that both diet and gill raker morphology (length and distance between gill rakers) in adults change with seasonal prey availability. Unless individuals undergo major migrations throughout the lake, or experience strong selection, this would imply that gill raker structure is highly plastic in adults and can change with prey availability (Millet 2013; Delarue 2016). A study by (Bolnick and Araújo 2011) on gill raker length and spacing in wild freshwater stickleback from British Columbia, Canada concluded that selection acts on sticklebacks' resource use. Consequently, selection pressures could favour differing phenotypes in Mývatn stickleback across developmental stages, seasons, and years in response to substantial fluctuations in resource availability.

2.5.4 Conclusions

This is one of the few studies to describe juvenile feeding ecology in wild freshwater stickleback, providing valuable insight into what the diet of wild juveniles consists of and how environment impacts diet composition. We found that both diet and head morphology are associated with habitat and body size. Especially the shift from small Cladocera to a more Chironomidae based diet was reflected in size and head shape. This is in line with findings of juvenile diet in other systems across the northern hemisphere, showing there is a general pattern of ontogenetic niche shift that shapes early development in threespine stickleback. Studying what shapes phenotypic variation at various developmental stages is crucial to identifying sensitive life stages. This could become even more important as environmental conditions become progressively unpredictable due to climate change.

Acknowledgements

We thank all field volunteers who have helped to collect the data presented in this paper. Special thanks go to Kasha Strickland and Joseph Phillips that supported us in sample collection and processing. These surveys were conducted under the auspices of the Nature Research Centre at Mývatn, which has government approval for collecting fish specimens from the lake. We also thank all landowners for allowing us to conduct research on their properties. Finally, we also thank Katja Räsänen for her support in writing this paper. Finally, this study was supported by the Icelandic Research Fund, grant of excellence (1955 71-052).

References

Amundsen P-A, Bøhn T, Popova OA, Staldvik FJ, Reshetnikov YS, Kashulin NA, Lukin AA. 2003. Ontogenetic niche shifts and resource partitioning in a subarctic piscivore fish guild. Hydrobiologia. 497(1):109–119.

Bartrons M, Einarsson Á, Nobre RLG, Herren CM, Webert KC, Brucet S, Ólafsdóttir SR, Ives AR. 2015. Spatial patterns reveal strong abiotic and biotic drivers of zooplankton community composition in Lake Mývatn, Iceland. Ecosphere. 6(6):art105.

Bell MA. 1981. Lateral plate polymorphism and ontogeny of the complete plate morph of threespine sticklebacks (*Gasterosteus aculeatus*). Evolution. 35(1):67–74.

Berner D, Adams DC, Grandchamp A-C, Hendry AP. 2008. Natural selection drives patterns of lake-stream divergence in stickleback foraging morphology. J Evol Biol. 21(6):1653–1665.

Bolnick DI, Araújo MS. 2011. Partitioning the relative fitness effects of diet and trophic morphology in the threespine stickleback. Evol Ecol Res. 13(5):439–459.

Bolnick DI, Ballare KM. 2020. Resource diversity promotes among-individual diet variation, but not genomic diversity, in lake stickleback. Ecol Lett. 23(3):495–505.

Cresko WA, McGuigan KL, Phillips PC, Postlethwait JH. 2007. Studies of threespine stickleback developmental evolution: progress and promise. Genetica. 129(1):105–126.

Crichigno SA, Battini MÁ, Cussac VE. 2014. Diet induces phenotypic plasticity of *Percichthys trucha* (Valenciennes, 1833) (Perciformes, Percichthyidae) in Patagonia. Zool Anz. 253(3):192–202.

Day T, McPhail JD. 1996. The effect of behavioural and morphological plasticity on foraging efficiency in the threespine stickleback (*Gasterosteus sp.*). Oecologia. 108(2):380–388.

Day T, Pritchard J, Schluter D. 1994. A comparison of two sticklebacks. Evolution. 48(5):1723–1734.

Delarue C. 2016. Plasticity of trait divergence in threespine stickleback (*Gasterosteus aculeatus*) from Lake Mývatn. Kristjánsson B, Räsänen K, editors. MSc Thesis, Hólar University.

Delbeek JC, Williams DD. 1988. Feeding selectivity of four species of sympatric stickleback in brackish-water habitats in eastern Canada. J Fish Biol. 32(1):41–62.

Demchuk A, Ivanov M, Ivanova T, Polyakova N, Mas-Martí E, Lajus D. 2015. Feeding patterns in seagrass beds of three-spined stickleback *Gasterosteus aculeatus* juveniles at different growth stages. J Mar Biol Assoc UK. 95(8):1635–1643.

Einarsson Á, Örnólfsdóttir EB. 2004. Long-term changes in benthic Cladocera populations in Lake Myvatn, Iceland. Aquat Ecol. 38(2):253–262.

Einarsson Á, Stefánsdóttir G, Jóhannesson H, Ólafsson JS, Már Gíslason G, Wakana I, Gudbergsson G, Gardarsson A. 2004. The ecology of Lake Myvatn and the River Laxá: Variation in space and time. Aquat Ecol. 38(2):317–348.

Garduno-Paz MV, Couderc S, Adams CE. 2010. Habitat complexity modulates phenotype expression through developmental plasticity in the threespine stickleback. Biol J Linn Soc Lond. 100(2):407–413.

Gill AB, Hart PJB. 1994. Feeding behaviour and prey choice of the threespine stickleback: the interacting effects of prey size, fish size and stomach fullness. Anim Behav. 47(4):921–932.

Gisbert E, Merino G, Muguet JB, Bush D, Piedrahita RH, Conklin DE. 2002. Morphological development and allometric growth patterns in hatchery-reared California halibut larvae. J Fish Biol. 61(5):1217–1229.

Hangelin C, Vuorinen I. 1988. Food selection in juvenile three-spined sticklebacks studied in relation to size, abundance and biomass of prey. Hydrobiologia. 157(2):169–177.

Hendry AP, Peichel CL, Matthews B, Boughman JW, Nosil P. 2013. Stickleback research: the now and the next. Evol Ecol Res. 15(2):111–141.

Hendry AP, Taylor EB, McPhail JD. 2002. Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the Misty system. Evolution. 56(6):1199–1216.

Hjelm J, van de Weerd GH, Sibbing FA. 2003. Functional link between foraging performance, functional morphology, and diet shift in roach (*Rutilus rutilus*). Can J Fish Aquat Sci. 60(6):700–709.

Hynes HBN. 1950. The food of fresh-water sticklebacks (*Gasterosteus aculeatus* and *pygosteus pungitius*), with a review of methods used in studies of the food of fishes. J Anim Ecol. 19(1):36.

Ingram T, Svanbäck R, Kraft NJB, Kratina P, Southcott L, Schluter D. 2012. Intraguild predation drives evolutionary niche shift in threespine stickleback. Evolution. 66(6):1819–1832.

Ives AR, Einarsson Á, Jansen VAA, Gardarsson A. 2008. High-amplitude fluctuations and alternative dynamical states of midges in Lake Myvatn. Nature. 452(7183):84–87.

Jonsson B, Jonsson N. 2014. Early environment influences later performance in fishes. J Fish Biol. 85(2):151–188.

Kimirei IA, Nagelkerken I, Trommelen M, Blankers P, van Hoytema N, Hoeijmakers D, Huijbers CM, Mgaya YD, Rypel AL. 2013. What Drives Ontogenetic Niche Shifts of Fishes in Coral Reef Ecosystems? Ecosystems. 16(5):783–796.

Kristjánsson BK. 2001. Divergence of Icelandic threespine stickleback, *Gasterosteus aculeatus*, to two bottom types in lakes and recently formed lagoon. Noakes DLG, Skulason S, editors. MSc Thesis, The University of Guelph.

Kristjánsson BK, Skúlason S, Noakes DL. 2002. Morphological segregation of Icelandic threespine stickleback (*Gasterosteus aculeatus L.*). Biol J Linn Soc Lond. 76(2):247–257.

Matthews B, Marchinko KB, Bolnick DI, Mazumder A. 2010. Specialization of trophic position and habitat use by sticklebacks in an adaptive radiation. Ecology. 91(4):1025–1034.

McGee MD, Schluter D, Wainwright PC. 2013. Functional basis of ecological divergence in sympatric stickleback. BMC Evol Biol. 13:277.

Millet A. 2013. Spatio-temporal variation of threespine stickleback (*Gasterosteus aculeatus*) in the dynamic Lake Mývatn, Iceland. Kristjánsson BK, Räsänen K, editors. PhD thesis, University of Iceland.

Millet A, Kristjánsson BK, Einarsson Á, Räsänen K. 2013. Spatial phenotypic and genetic structure of threespine stickleback (*Gasterosteus aculeatus*) in a heterogeneous natural system, Lake Mývatn, Iceland. Ecol Evol. 3(10):3219–3232.

Moosmann M, Hudson CM, Seehausen O, Matthews B. 2022. The phenotypic determinants of diet variation between divergent lineages of threespine stickleback. Evolution. 77(1): 13–25.

Nonaka E, Svanbäck R, Thibert-Plante X, Englund G, Brännström Å. 2015. Mechanisms by Which Phenotypic Plasticity Affects Adaptive Divergence and Ecological Speciation. Am Nat. 186(5):E126-43.

Ólafsson J. 1979. Physical Characteristics of Lake Mývatn and River Laxá. Oikos. 32(1/2):38–66.

Osse JWM, van den Boogaart JGM, van Snik GMJ, van der Sluys L. 1997. Priorities during early growth of fish larvae. Aquaculture. 155(1):249–258.

Phillips JS, Einarsson Á, Strickland K, Ives AR, Kristjánsson BK, Räsänen K. 2023. Demographic Basis of Spatially Structured Fluctuations in a Threespine Stickleback Metapopulation. Am Nat. 201(3):E41–E55.

Phillips JS, McCormick AR, Einarsson Á, Grover SN, Ives AR. 2019. Spatiotemporal variation in the sign and magnitude of ecosystem engineer effects on lake ecosystem production. Ecosphere. 10(6):e02760.

Posit team. 2023. RStudio: Integrated Development for R. http://www.posit.co/.

Reimchen TE. 1990. Size-Structured Morality in a Threespine Stickleback (*Gastrosteus aculeatus*) – Cutthroat Trout (*Oncorhynchus clarki*) Community. Can J Fish Aquat Sci. 47(6):1194–1205.

Robinson B. 2000. Trade offs in habitat-specific foraging efficiency and the nascent adaptive divergence of sticklebacks in lakes. Behaviour. 137(7–8):865–888.

Rudolf VHW. 2020. A multivariate approach reveals diversity of ontogenetic niche shifts across taxonomic and functional groups. Freshw Biol. 65(4):745–756.

Schluter D. 1995. Adaptive radiation in sticklebacks: trade-offs in feeding performance and growth. Ecology. 76(1):82–90.

Seehausen O, Wagner CE. 2014. Speciation in Freshwater Fishes. Annu Rev Ecol Evol Syst. 45(1):621–651.

Skúlason S, Noakes DLG, Snorrason SS. 1989. Ontogeny of trophic morphology in four sympatric morphs of arctic charr *Salvelinus alpinus* in Thingvallavatn, Iceland. Biol J Linn Soc Lond. 38(3):281–301.

Skúlason S, Parsons KJ, Svanbäck R, Räsänen K, Ferguson MM, Adams CE, Amundsen P-A, Bartels P, Bean CW, Boughman JW, et al. 2019. A way forward with eco evo devo: an extended theory of resource polymorphism with postglacial fishes as model systems. Biol Rev Camb Philos Soc. 94(5):1786–1808.

Snorradóttir RG. 2023. Relationships between phenotype, diet, and individual specialization of threespine stickleback. Kristjánsson BK, editor. MSc thesis, Hólar University.

Strickland K, Matthews B, Jónsson ZO, Kristjánsson BK, Phillips JS, Einarsson Á, Räsänen K. 2024. Microevolutionary change in wild stickleback: Using integrative time-series data to infer responses to selection. Proc Natl Acad Sci USA. 121(37):e2410324121.

Strickland K, Räsänen K, Kristjánsson BK, Phillips JS, Einarsson Á, Snorradóttir RG, Bartrons M, Jónsson ZO. 2023. Genome-phenotype-environment associations identify signatures of selection in a panmictic population of threespine stickleback. Mol Ecol. 32(7):1708–1725.

West-Eberhard MJ. 1989. Phenotypic Plasticity and the Origins of Diversity. Annu Rev Ecol Syst. 20(1):249–278.

Wund MA, Baker JA, Clancy B, Golub JL, Foster SA. 2008. A Test of the “Flexible Stem” Model of Evolution: Ancestral Plasticity, Genetic Accommodation, and Morphological Divergence in the Threespine Stickleback Radiation. Am Nat. 172(4):449–462.

3 Plasticity in key life history traits of threespine stickleback (*Gasterosteus aculeatus*) in response to temperature and diet

Alessandra Schnider [1,2], Katja Räsänen [3], Kasha Strickland [4], Joseph S. Phillips [5], Bjarni K. Kristjánsson [1]

[1] Department of Aquaculture and Fish Biology, Hólar University, Iceland
[2] Faculty of Life and Environmental Sciences, University of Iceland, Iceland
[3] Department of Biological and Environmental Science, University of Jyväskylä, Finland
[4] Institute of Ecology and Evolution, School of Biological Sciences, University of Edinburgh, UK
[5] Department of Biology, Creighton University, Omaha, NE 68178, USA

3.1 Abstract

Natural selection targets individuals' phenotype, which can be influenced by phenotypic plasticity, within and across generations. When plasticity is adaptive, it can enable organisms to rapidly respond to varying environmental conditions and maximise their fitness. Experiments studying this have predominantly focused on early developmental stages and isolated environmental variables. Hence, how interactions between different environmental variables affect phenotypic variation within and across generations is still not fully understood. Here we present data from a multigenerational plasticity experiment, where we tested the effects of contrasting temperatures and diets and their interaction on key life history traits, such as body size, maternal investment, and embryonic survival. To this end, offspring of threespine stickleback originating from two contrasting thermal habitats within lake Mývatn ("Cold Shore" vs. "Hot Shore") were reared in the laboratory in two contrasting temperatures (12°C vs. 22°C) and two dietary conditions (benthic vs. epibenthic) across two generations (F1 & F2). We recorded egg and clutch size, fertilization and hatching success, juvenile and female size (weight), and condition at maturity.

For the F1 generation, we found that females reared at 12°C on an epibenthic diet were smaller and produced fewer, but larger eggs compared to females reared at 22°C on a benthic diet. Furthermore, eggs of F1 females with a Hot Shore origin had a higher fertilization and hatching success at 22°C. For the F2 generation, we found that juveniles were heavier if either the parents or they themselves were reared on an epibenthic diet. However, at maturity, the F2 females were larger when reared at 12°C and on a benthic diet. We found limited effects of origin on the measured traits, but substantial evidence for parental and grandparental plasticity on key life history traits in Mývatn threespine

stickleback. Our findings stress the varying impact that phenotypic plasticity, including transgenerational effects, can have on different fitness components across the lifetime of organisms. Finally, this study highlights the importance of multigenerational experiments to gain understanding on the interaction of environment and phenotype.

3.2 Introduction

An individual's phenotype is the result of the interaction between its genes and the environment (Houle et al. 2010). Phenotypic plasticity, defined as the differential expression of organism's phenotype in response to environmental stimuli, is common in systems where conditions change rapidly over space and/or time, facilitating adaptive plasticity if environmental variation is predictable (Sultan and Spencer 2002; Fawcett and Frankenhuis 2015) and costs of phenotypic plasticity low (Pfennig 2021). Moreover, an individual's phenotype can also be impacted by the parental and grandparental environment (Danchin et al. 2011). If environmental cues are reliable across generations, the parental phenotype can "inform" the offspring's phenotype, in so called adaptive transgenerational effects (Pfennig 2021), further facilitating rapid phenotypic responses to changing environments.

Plasticity in life history traits can have significant fitness implications. According to life history theory, individuals face trade-offs that require them to optimize energy allocation between growth and reproduction. Additionally, they must balance energy investment across different components of reproduction, such as egg size and egg number, to maximize their lifetime fitness (Roff 1994; Stearns 1998; Kaplan and Robson 2009). Therefore, plasticity in life history traits can have significant fitness implications. For instance, clutch mass is often a function of female body size, as larger females are less physically constrained in how much of their body can be allocated to producing and storing eggs (Wootton 1977; Baker et al. 2015; Kasimatis and Riginos 2016). Thus, if a female grows bigger, she is able to produce a bigger clutch, and by that likely increase her fitness. However, as a female is limited not only in space but also in energy that she can allocate to reproduction, she can modulate egg number and size. Often females in adverse conditions, such as poor food availability or quality, will rather invest in fewer but bigger eggs, if the offspring is likely to encounter similar conditions (Mousseau and Fox 1998a; Ernsting and Isaaks 2000; Shama 2015). Nevertheless, we still have not fully understood how varying environmental conditions may affect the reproductive efforts of females and if and how any variation in female investment may affect juvenile fitness.

In oviparous species, transgenerational effects are often maternal and mediated through egg size and composition (Mousseau and Fox 1998a; Räsänen and Kruuk 2007; Moore et al. 2019; Marks and Lailvaux 2024). Egg size has often been found to be plastic in response to varying environmental conditions. Egg size plasticity can reflect differential resource availability, but also different maternal investment strategies (Mousseau and Fox 1998b). Under adverse conditions, such as poor food availability or quality, investment in fewer but larger eggs may be favoured (Mousseau and Fox 1998a; Ernsting and Isaaks 2000; Shama 2015).

Some environments pose especially strong challenges, eliciting strong phenotypic responses. For example, spadefoot toads (*Spea multiplicate*) inhabit desert environments where tadpoles develop in temporary pools formed by rare rainfalls and need to complete

development before the ponds dry up. Based on environmental cues tadpoles develop into two varying ecomorphs with respective physical and behavioural adaptations (Levis et al. 2015). Phenotypic plasticity has been studied across a wide range of taxa and environmental conditions (West-Eberhard 1989; Pigliucci 2005; Del Giudice 2015; Metcalfe 2024). A multitude of studies have focused on the effects of elevated temperatures (Jonsson and Jonsson 2014; Shama et al. 2014; Potts et al. 2021) and diet (Meyer 1987; Gunter et al. 2013; Levis et al. 2015; Bolnick and Ballare 2020). However, not much is known of their interaction, and how these potential interactions affect key life history components, such as maternal investment, embryonic survival, as well as juvenile and adult size within and across generations.

In ectotherms temperature can have an especially large effect, impacting metabolic rate and the pace of development, growth and body size, as well as fecundity, age at maturation and fitness (Sibly and Atkinson 1994; Pörtner et al. 2001; Angilletta et al. 2004; Georga and Koumoundouros 2010; Ramler et al. 2014). Moderate increases in temperature can have beneficial effects, such as increased growth and early maturation (Zuo et al. 2012), whereas large increases in temperature can result in stress responses, such as reduced reproductive success and oxidative damage (Boulé and Fitzgerald 1989; Pörtner et al. 2001; Carney Almroth et al. 2015; Ritchie and Friesen 2022).

Diet is another important component of the environment that can strongly affect life history and shape phenotypic variation (Schluter 1996). In fish, a common axis of divergence is between benthic diet (feeding on or close to the ground) and pelagic diet (feeding in the water column) evidenced in the evolution of resource polymorphism (Seehausen and Wagner 2014; Skúlason et al. 2019). These two foraging strategies are known to promote divergence in overall body shape, gill raker number and structure, and gut length both through genetic divergence and phenotypic plasticity (Day et al. 1994; Cresko et al. 2007; Crichigno et al. 2014). Moreover, diet can alter phenotypic variation through developmental plasticity, as observed in various laboratory experiments, where fish reared either on the same or contrasting diet consequently developed similar or diverging phenotypes (Hegrenes 2001; Gunter et al. 2013; Crichigno et al. 2014; Delarue 2016).

Despite the extensive studies on the long-term effects of elevated temperatures and contrasting diet on phenotypic plasticity on various traits, not much is known so far of their interaction and how these potential interactions affect reproductive success and potential transgenerational effects. The goal of this study was to investigate the combined effects of temperature and diet on key life history traits, including embryonic survival, juvenile size, female size at maturity and maternal investment (egg size and number), across multiple generations of threespine stickleback (*Gasterosteus aculeatus*).

Here we tested the effects of parental environment, temperature and diet on embryos, juveniles, and sexually mature females within and across generations. We conducted a laboratory rearing experiment across two generations where offspring of threespine stickleback originating from two contrasting habitats ("Cold Shore": cold temperatures and mostly epibenthic diet; "Hot Shore": warm temperature and mostly benthic diet (Millet 2013)) within Lake Mývatn, Iceland (Fig. 3.1) were reared at two contrasting temperatures (12°C and 22°C) and on two diets (benthic vs. epibenthic) (Fig. 3.2). Threespine stickleback from Mývatn, are well-suited to study phenotypic plasticity and transgenerational effects in response to temperature and diet variation. This is because

Mývatn encompasses substantial temporal and spatial variation in temperature and invertebrate communities (Einarsson et al. 2004; Ives et al. 2008; Bartrons et al. 2015). Further, previous work has found phenotypic divergence among habitats, with subtle genetic divergence (Kristjánsson et al. 2002; Ólafsdóttir et al. 2007; Millet et al. 2013; Strickland et al. 2023).

We made the following main predictions: First, we would expect offspring in environments matching parental environment to do better than in contrasting conditions. Especially so in the warm temperature treatment, as acclamation to warm temperatures has been shown to be costly. Second, if there is temperature or diet induced plasticity within generations, we would expect phenotypic changes in traits such as body size and maternal investment. Finally, if there is temperature or diet induced transgenerational plasticity, we would expect the individuals to exhibit phenotypic changes reflecting the environmental conditions of their parental or even grandparental generation, independent of their current conditions.

3.3 Methods

3.3.1 Study system

Mývatn (65°36'N, 17°00'W; 278m a.s.l.) in North-Eastern Iceland is a shallow eutrophic lake that consists of two main basins (North and South basin) and varies greatly in a range of abiotic and biotic factors across space and time (Fig. 3.1) (Einarsson et al. 2004). The lake is spring fed, with both cold-water springs (~5°C) in the south-east and geothermal hot springs (up to ~23°C) in the north-east, which create temperature gradients along the eastern shore, whereas temperatures in other parts of the lake are mostly subjected to seasonal temperature fluctuations (Johansson et al. 2016). Furthermore, the lake varies greatly in productivity and invertebrate communities, in space and time, being dominated by Chironomidae and Cladocera, which constitute the primary food source of adult stickleback (Einarsson and Örnólfsdóttir 2004; Ives et al. 2008; Millet et al. 2013; Bartrons et al. 2015).

Five types of stickleback habitats ("Warm", "Mined", "Rocky Shore", "Cladophorales" and "Pondweed"), have been described in Mývatn (Millet et al. 2013) (Fig. 3.1), based on depth, substrate, vegetation, and temperature. For the current study, we used fish originating from the "Warm" habitat ("Hot Shore", henceforth HS) in the north-east of the North basin, and "Shore" habitat ("Cold Shore", henceforth CS) in the north-west of the South basin. The HS habitat has an average year-round water temperature of 20-23°C whereas CS habitat, the water temperature follows the ambient temperature seasonally, with average summer temperature 11-13°C (Ólafsson 1979; Millet et al. 2013). Although the strongest contrast experienced by stickleback between HS and CS environments is temperature, the diet of stickleback also differs with HS individuals tending to have relatively more Chironomidae (benthic prey) and CS individuals relatively more Cladocera (epibenthic prey) in their diet (Millet 2013).

3.3.2 Collection of breeding fish

This study involves the lethal sampling of wild animals, bringing living animals to rearing facilities and subjecting them to experimental conditions. All experimental work was done in accordance with Icelandic laws and regulations set forward by the Icelandic animal care authorities (animal care permit number: 2020-03-02). To set up the multigenerational experiment, we collected males and females in reproductive condition from the HS and CS in June 2020 using unbaited minnow traps, placed overnight (~12 hours at each site). Upon collection, males with nuptial colouration and females with clearly developing eggs were transported in well oxygenated tanks from Mývatn to the research facilities at Verið, the main facilities of the Department of Aquaculture and Fish Biology of Hólar University in Sauðárkrókur. In the rearing lab, the wild caught adults were sorted by sex and origin and maintained in 27L buckets in a custom-made flow through system. The fish were maintained at approximately 18°C and under 24 hours light (reflecting an intermediate temperature of the two sites and Icelandic summer light conditions) and fed frozen midge (Chironomidae) larvae twice a day until crosses were made (see below).

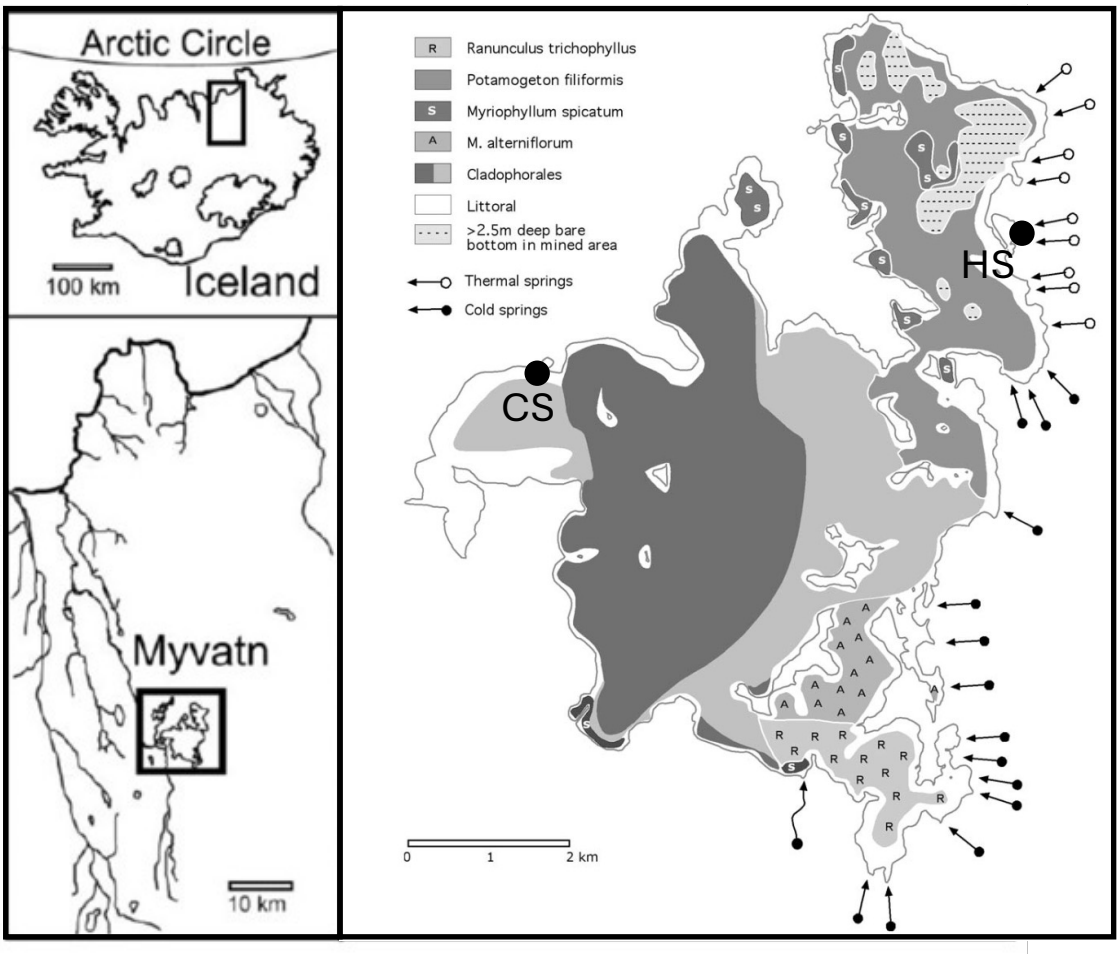

Figure 3.1 *Map of Lake Mývatn, NE Iceland, depicting the dominant benthic macrophytes (in late 2000) and the main spring water inflows. The two shades of grey used to describe the Cladophorales habitats reflect density (lighter: less dense, darker: denser). Black circles mark the two sampling sites of threespine stickleback representing the two habitats "Cold Shore" (labelled "CS") and "Hot Shore" (labelled "HS"). Edited from Einarsson et al. (2004).*

3.3.3 Experimental design

The experimental design consisted of rearing offspring of the wild collected parents (P) from the two contrasting thermal origins (HS and CS) at two contrasting temperatures (cold: 12°C vs. warm: 22°C) and two contrasting diets (benthic vs. epibenthic) across F1 and F2 generations (Fig. 3.2). Experimental temperatures were kept constant across the F1 and F2 generations, whereas diet of the F2 generation was either matching or contrasting the diet of the F1 parental generation (Fig. 3.2). To create different sibships at each generation, the fish were artificially crossed using standard procedures (for crossing details see below). Upon artificial crosses, both F1 and F2 fish were reared until sexual maturity. To test the effects of temperature and diet both within and across generations, a subset of F1 and F2 individuals were weighed and photographed at juvenile and adult life stages (details below).

3.3.4 Crossing protocols

Prior to artificial crosses of the P or F1 generation fish, females were checked daily to assess if any were ready to spawn (identified by a round abdomen and swollen cloaca, which allowed us to see the first egg). The most colourful males in a tank were chosen, as breeding colouration reflects breeding condition (Candolin 1999). At the start of crossing, the male was euthanized by placing it in a 2-phenoxyethanol solution (Priborsky and Velisek 2018). Subsequently, the testes were dissected out and crushed in either a small petri dish with tap water (P generation) or Ginsburg's solution (F1 generation, see below).

The female was weighed before stripping and without aesthesia to avoid contact between the eggs and phenoxyethanol. Once weighed the female's abdomen was gently massaged from the centre of the body towards the cloaca to release the eggs. When all the eggs were released, the female was weighed again, to determine clutch weight and clutch free body weight. Finally, the female was euthanized by placing it in a strong 2-phenoxyethanol solution and a photograph of their left side was taken (Priborsky and Velisek 2018). The egg and sperm solution were gently mixed and left to rest for 30 minutes, after which all the water and the testis were removed, and fresh water added. Approximately one hour post fertilization (egg hardening phase in stickleback (Swarup 1958)), the formation of a zygote was visually confirmed under a dissecting microscope to assure that fertilization was successful.

To create the F1 generation, a total of 34 full sibling families (CS: N = 18, HS: N = 16) were created through artificial crossing over the course of two weeks in June 2020. Once fertilization was verified, the F1 clutches were carefully split in half with a small brush and photographed to assess fertilization and hatching success. Next the two halves of the F1 clutches were randomly assigned to one of the two temperature treatments (cold: 12°C vs. warm 22°C).

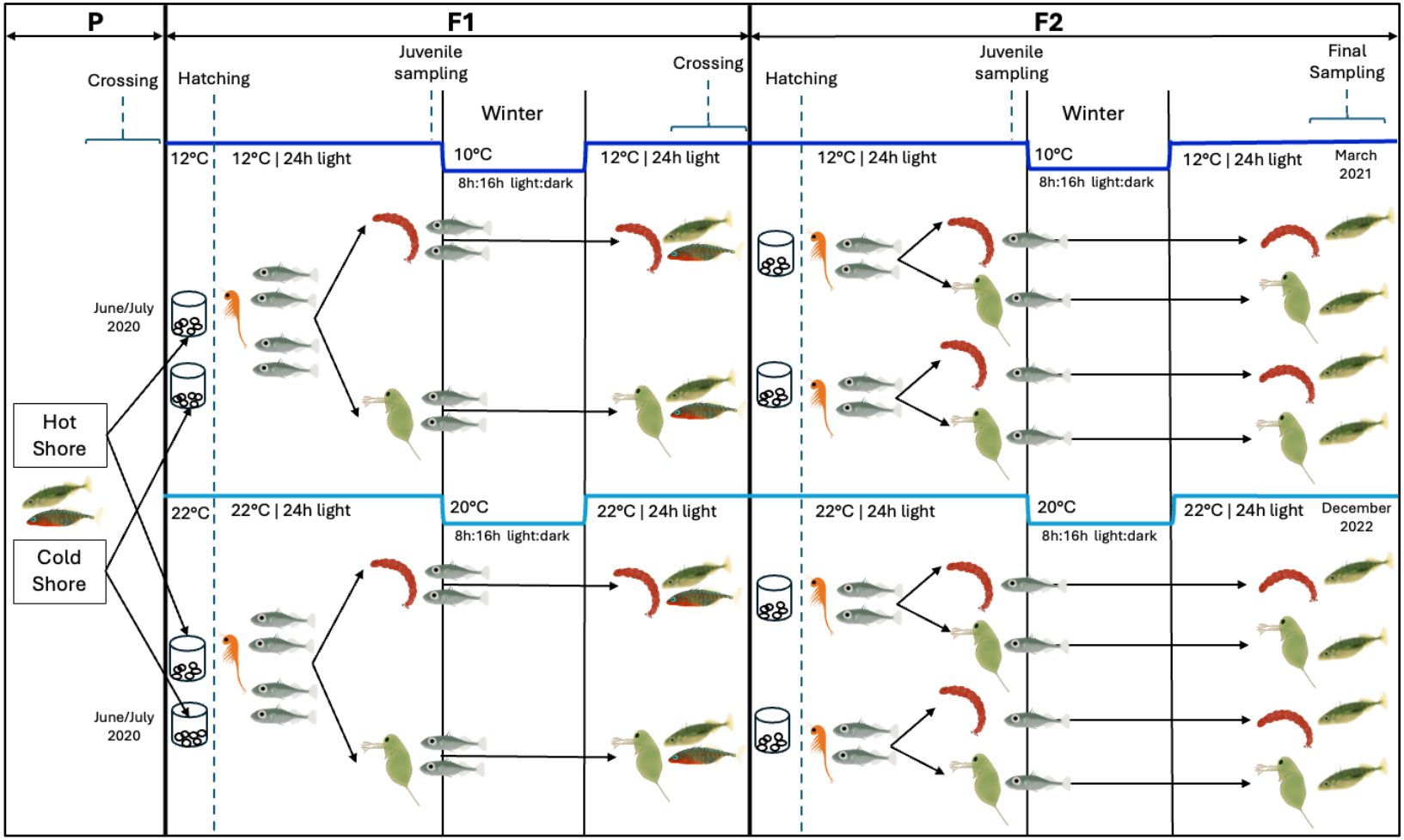

Figure 3.2 *Illustration of the design of a transgenerational plasticity experiment conducted on threespine stickleback (Gasterosteus aculeatus) from Lake Mývatn Iceland, originating from two locations "Hot Shore" and "Cold Shore" within the lake. It includes illustrations of fish at juvenile and adult sampling stage as well as food items used in this experiment (orange: Artemia salina; red: Midge (Chironomidae) larvae; green: Cladocera spp.). Artemia salina were used post hatching until the experimental fish were split into their respective diet treatments; benthic diet: Midge larvae and epibenthic diet: Cladocera spp. supplemented with Artemia salina. It also depicts the light and temperature regime across the whole experimental period as well as sampling events*

To reduce genetic variation among individuals within treatment combinations, and hence focus on phenotypic plasticity, we aimed to produce inbred family crosses (i.e. conduct crosses between full siblings) for the F2 generation. Based on the low inbreeding coefficients (F) of Mývatn stickleback (Strickland et al. 2023), we did not expect substantial inbreeding depression within this generation. However, due to low fertilization success in the warm treatment, we had to also include outbred crosses (crosses between unrelated individuals from the same parental origin, i.e. CS vs. HS). To increase fertilization success in the warm treatment, we also adjusted the crossing protocol itself (with the previous protocol fertilization in the warm temperature treatment was not successful): instead of tap water, the testes were crushed in a Ginsburg's solution in a 2ml Eppendorf tube. The Ginsburg's solution is a saline solution that increases the fertilization window by prolonging sperm activity (Lahnsteiner 2011). Additionally, by using a 2ml Eppendorf tube, the volume of the solution relative to sperm was reduced, increasing the likelihood of sperm encountering eggs. Eggs were stripped into the Eppendorf tube and left in the sperm solution for 10 min. Thereafter, the eggs were transferred into a small petri dish, tap water was added, and eggs treated as during crossing of the P generation.

In March/April 2021, a total of 47 F2 crosses were created in the warm treatment (22 benthic, 25 epibenthic) over the course of nine days. In May/June 2021, a total of 37 crosses were created in the cold treatment (17 benthic, 20 epibenthic), over the course of four days. We had exclusively full sibling crosses in the cold treatment, whereas in the warm treatment we had both within ("inbred") and between ("outbred") family crosses (benthic: 10 inbred, 3 outbred; epibenthic: 9 inbred, 9 outbred). Once enough successful crosses were obtained for each origin treatment combination, all remaining adults were terminally sampled and processed as in F1 generation (see above).

3.3.5 Rearing conditions and juvenile sampling

As temperature is one of the major determinants of developmental trajectories in fish (Ramler et al. 2014), we standardized diet transition and juvenile sampling dates based on degree days (sum of daily temperatures) (Fig. 3.2), i.e. to sample individuals at comparable developmental stages rather than matching calendar days. Although, the relationship between developmental trajectories and temperature is not linear (Ramler et al. 2014), using degree days allowed to correct for some of the differences in developmental speed due to contrasting experimental temperatures. For rearing, the embryos were placed in a breeding cup (plastic cup: 9.5cm high, 7.4cm diameter; with mesh bottom: 1mm mesh size) floating in a 13L tank (33 x 21 x 19 cm) and reared in a flow-through system at the respective treatment temperature. Eggs were checked daily, and dead eggs counted and removed.

Shortly after most juveniles in a breeding cup reached the swim-up stage (yolk sac is absorbed and fish start actively swimming and foraging), all individuals were counted and released from the cups to swim freely in their 13L rearing tank. All individuals were fed live *Artemia salina* nauplii *ad libitum* twice a day, seven days a week. After approximately 460 degree-days (i.e. two weeks in warm and four weeks in cold rearing temperatures), fish were big enough to be transitioned to either benthic or epibenthic diet treatments (Fig. 3.2). At this point, the group of individuals from each tank were split in half and randomly assigned to one of the diet treatments. Each tank contained 9-35 individuals of the same family in the F1 generation. In the F2 generation each tank held 7-25 individuals of one to

three families in the F2 generation. Furthermore, each tank was enriched with an artificial plant (black plastic bag wrapped around a rock) and two petri dishes filled with sand.

The fish in the epibenthic treatment were fed a mix of live Cladocera (*Chydorus sphaericus* and *Daphnia sp.*, cultured in our research facilities, at the water surface so they could fall through the water column, whereas fish in the benthic treatment were fed commercially available frozen midge larvae (Chironomidae midges, *Ruto Frozen Fishfood*) in the sand dish at the bottom of the tank. To ensure that small juveniles were able to ingest midge larvae, these were finely grated or chopped until fish were large enough to ingest entire midge larvae. Furthermore, as it was not possible to produce enough Cladocera to sustain all the fish on a pure epibenthic diet, we supplemented the epibenthic treatment with *Artemia salina* nauplii as needed. To ensure that fish received all the essential nutrients, all juveniles additionally received aquaculture pellets once a week (INICIO Plus G by BioMar), starting with 0.4mm pellet size and as fish size increased, pellet size was increased to 0.5mm. The pellets were composed of crude protein (62%), crude fat (10%), crude fibre (0.2%), ash (10.3%), phosphorus (1.53%), calcium (2.16%), sodium (0.75%). Pellets were either fed at the water surface or in the petri dishes, matching the diet treatment.

We set the juvenile sampling at c. 1300 degree-days (60 days in warm and 110 days in cold rearing temperatures), because previous studies have shown that by that time, skeletal development is complete (Currey et al. 2017). In the F1 generation, a total of 943 juveniles in the cold and 1219 juveniles in the warm were weighed and returned to the 13L rearing tank (Table 3.1). Furthermore, some randomly selected individuals were removed to reduce tank density to max. 20 individuals per tank (i.e. no more than 2 fish per litre). Two weeks after the 1300 degree-day juvenile sampling, fish were transitioned to winter conditions for four months in both temperature treatments. During winter conditions, temperatures were reduced to 10°C and 20°C, respectively, and a photoperiod of 8h:16h light:dark (Fig. 3.2). To adjust for lower metabolic demands at these lower "winter" temperatures, feeding with treatment diet (Cladocera vs. midge larvae) was reduced to four days a week (total of 7 feeds), with pellets provided twice a week and a fasting day introduced, where fish did not receive any food.

After the four-month artificial winter period, the F1 fish were transitioned back to 24h light and 12°C vs. 22°C temperatures, to stimulate and synchronize sexual maturation. Feeding was increased to three days live feeds and three pellet feeds a week, whilst still including a fasting day, to meet the increased energetic demands due to higher temperatures. Further, an additional artificial plant was added to each tank to reduce aggression among males during sexual maturation, and the sand dishes removed to discourage males from building nests. Once most of the tanks contained gravid females and males displaying breeding colouration, crossing of the F2 generation commenced (details below). To assess maternal investment and female body size, all sexually mature F1 females in the warm treatment (benthic diet N = 59; epibenthic diet N = 59) were sampled in March/April 2021 (~9 months post fertilization) over a time span of 53 days, and all F1 females in the cold treatment (benthic diet N = 59; epibenthic diet N = 58) in May/June 2021 (~11 months post fertilization) over a time span of 18 days (Table 3.1).

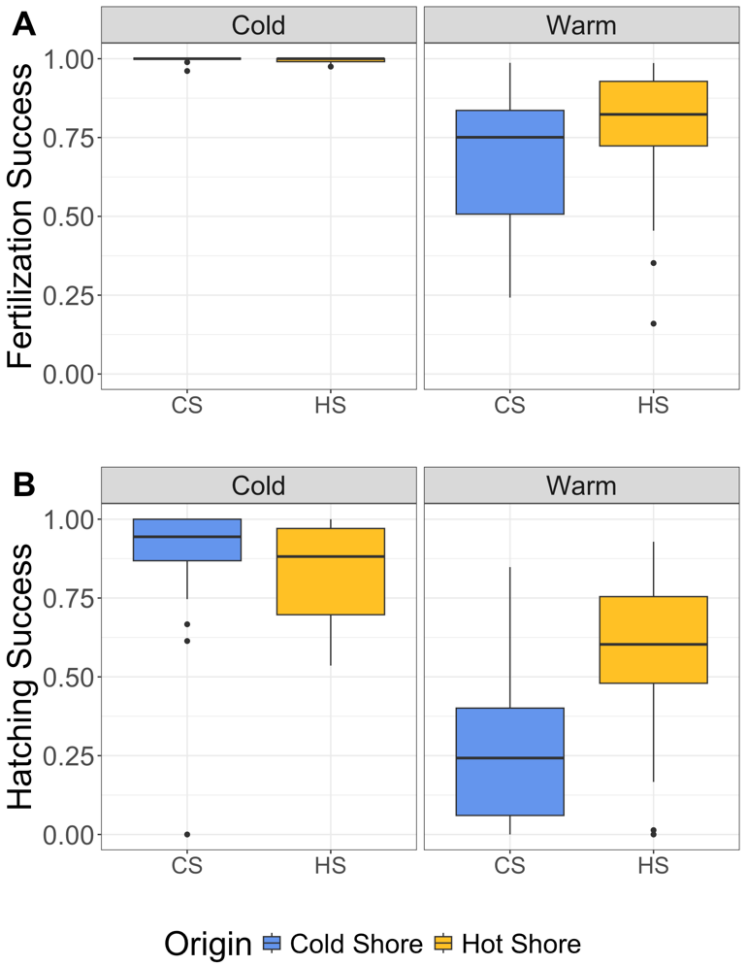

Figure 3.3 *Fertilization and hatching success of eggs produced by F1 female threespine stickleback reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic vs. epibenthic) (see Fig. 2). F1 Diet is not represented in this graph as we found no significant effect of diet neither on fertilization nor hatching success. Cold Shore (CS) and Hot Shore (HS) refer to the parental habitat within Lake Mývatn from which the P generation originated (Fig. 3.1). Single points represent uncorrected measurements of individual clutches. A) Fertilization success measured as the proportion of fertilized eggs per clutch obtained from F1 females. B) Hatching success measured as the proportion of fertilized eggs in a clutch that hatched. Boxplots show the median (horizontal line), interquartile range (box), and 1.5× interquartile range whiskers; each dot represent individual clutches outside this range (outliers).*

3.3.6 F2 generation

For the F2 generation, all offspring were reared in the same temperature treatment as their F1 parents (i.e. 22°C or 12°C; Fig. 3.2). Each clutch was incubated whole (i.e. not split) at a given temperature. To assess transgenerational effects induced by diet (within a given temperature), the F2 offspring were assigned (after two weeks in the warm and four weeks in the cold rearing temperature) to either a matching or contrasting parental diet (Fig. 3.2). At this point, offspring from each clutch were divided in half and reared on either benthic or epibenthic diet following the same procedures as for the F1 generation (Fig. 3.2).

However, due to low survival in the warm treatment (Fig. 3.3), 21 of the 32 families were at this point pooled to groups containing two to three families from the same grandparental origin (HS or CS), to reduce variation in body size due to contrasting tank densities. In the cold treatment, all individuals were reared in pure full-sibling groups after transition to their final diet treatment. After all F2 offspring were assigned to their diet treatments, each tank contained 7–25 individuals.

At c. 1300 degree-days, a total of 588 F2 juveniles in the warm and 947 in the cold (Table 3.2) were weighed, returned to their rearing tanks, and subsequently transitioned into an artificial winter period as in F1 (Fig. 3.2). After the four-month winter period, fish were transitioned back to summer conditions (Fig. 3.2). Once most of the tanks contained F2 females with developing eggs and males displaying breeding colouration, all adults were terminally sampled following the same sampling protocol as for the F1 generation. All F2 females in the warm treatment (N = 63) were sampled in December 2021 over a time span of 11 days, and those in the cold treatment (N = 95) in March 2022 over a time span of 8 days (Table 3.2).

3.3.7 Female size, maternal investment, and embryonic survival

To measure standard body length of mature females, a photograph of their left side was taken, either during the crossing protocol or during final sampling (see above), using a digital camera (*Canon EOS 750D* camera with a *Canon 50mm* macro lens mounted on a white light box to minimize shadows). The digital images were converted into *tps* files using the application *tpsUtil*. To measure body length, we used two fixed landmarks (subset of a total of 33 landmarks as in Snorradóttir (2023)) in *tpsDig2* v.2.32: one at the tip of lower lip and the other in the middle of the posterior end of vertebrae (McGee et al. 2013; Taugbøl et al. 2014). We performed a generalized Procrustes analysis using the "gpagen" function from the *geomorph* package (Baken et al. 2021) in *RStudio* (Posit team 2023) to rotate, scale and align landmarks. This produced four shape variables (X and Y coordinates for two landmarks) which were used in the "interlmkdist" function from the *geomorph* package to measure inter-landmark distance, producing standard length (hereafter SL) measurements for adult females. Further, female body condition (hereafter BC) was calculated with weight post stripping and standard body length using Fulton's condition factor (Nash et al. 2006).

To assess clutch size (number of eggs) and egg size, we used the digital pictures of the fertilized clutches taken one hour post fertilization (see above). We counted the total number of eggs per clutch, and the number of unfertilized eggs per clutch. Unfertilized eggs were identified by their opaque colouration and lack of a blastodisc. Hatching success was calculated as the proportion of hatched individuals relative to the number of fertilized eggs. To assess egg size of F1 females, we used ImageJ (Rasband, W.S. , U.S. National Institutes of Health, Bethesda, Maryland, USA 1997-2018). From each clutch, four diameters of 20 eggs per clutch were measured. All pictures were size standardized within ImageJ using the millimetre paper included in each picture. For statistical analyses, the mean diameter per egg was used. As the millimetre reference was missing in pictures of eggs produced by the wild caught P generation, eggs size was not assessed for that generation.

Table 3.1 *Sample size overview of first generation threespine stickleback of a plasticity experiment with individuals reared in contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic (midge larvae) vs. epibenthic (Cladocera)) (see Fig. 2). "Cold Shore" and "Hot Shore" refer to the parental (P) habitat within Lake Mývatn (Fig. 1). 18 Cold Shore crosses and 16 Hot Shore crosses were used to set up the F1 generation. Number of eggs and clutches refers to total eggs and crosses recorded during crossing of the F2 generation per treatment x origin combination. One clutch had to be excluded from hatching success in the Warm x Benthic x Cold Shore treatment x origin combination due to an inconsistency in the data collection.*

Temperature	*F1 Diet*	*Origin*	*F1 Juvenile weight*	*F1 female length*	*Nr. Eggs*	*Fertilization success Nr. clutches*	*Hatching success Nr. clutches*
Cold	**Benthic**	**Cold shore**	306	34	160	8	8
		Hot shore	151	25	180	9	9
	Epibenthic	**Cold shore**	310	31	240	12	12
		Hot shore	167	27	160	8	8
Warm	**Benthic**	**Cold shore**	387	30	290	15	14
		Hot shore	229	29	140	7	7
	Epibenthic	**Cold shore**	351	30	135	9	9
		Hot shore	252	29	295	16	16

3.3.8 Statistical analyses

All analyses and visualizations were done in *R* (R Core Team 2023). The data was analysed using general or generalized linear mixed models and simple linear models. All linear mixed-effects models were run using the "lmer" function from the *lme4* package (Bates et al. 2014). β and standard errors were extracted using the *base R* "summary" function. The linear and generalized linear models were run using the "lm" and "glm" function from the base *stat*s package, respectively (R Core Team 2023). Residual degrees of freedom, *F* and *p*-values were obtained with the "Anova" function (statistic = F and type = II) from the *car* package (Fox and Weisberg 2018). Residuals were extracted using the "resid" function from the *stats* package (R Core Team 2023).

Analyses of juvenile and adult female body size

We compared the standard length (SL) and body condition (BC) of wild-caught P females from different origins (CS vs. HS) using linear models with origin as a fixed effect. To test for the effects of origin and experimental treatments (temperature and diet) on the weight of F1 juveniles as well as SL and BC of F1 females, we used linear mixed-effects models. These models included origin (CS vs. HS), temperature (cold vs. warm), F1 diet (benthic vs. epibenthic) and all two-way interactions as fixed effects. To account for family and tank effects, we included tank identity nested within family identity as a random effect (family/tank ID). The final model structure used to test for treatment effects on F1 juvenile weight and F1 female SL and BC was: ~ Temperature* F1 Diet + Temperature * Origin + F1 Diet * Origin + (1|Family/Tank ID). We also tested for an effect of sampling date in data of the F1 females, due to the extended sampling period in the warm temperature treatment (warm: 53 days vs. cold: 18 days). However, we did not find any significant effect of sampling date on SL ($F = 0.11$, $p = 0.743$, Supplementary Fig. B.1) and thus, we did not include it in the final model.

To analyse F2 juvenile weight, we used a linear mixed model which included temperature (cold, warm), F1 and F2 diets (benthic vs. epibenthic), origin (CS vs. HS) and all two-way interactions. We also included tank identity as a random effect in the model to account for tank level effects. As multiple families were mixed within tanks in the warm treatment and individuals could not be traced back to a family identity, we could not correct for family in the F2 juveniles (see methods). Thus, the final model for F2 juvenile weight was: ~ Temperature * F1 Diet + Temperature * F2 Diet + Temperature + Origin + F1 Diet * F2 Diet + F1 Diet * Origin + F2 Diet * Origin + (1|Tank ID).

To analyse SL and BC of F2 females, we included rearing temperature (cold vs. warm) and F1 and F2 diets (benthic vs. epibenthic) as fixed effects. Due to relatively small sample size in the F2 females, we were not able to test for origin effects. We further reduced model complexity by only including the interactions between temperature and F2 diet as well as F1 and F2 diets. We also included tank identity as a random effect to accounted for tank level effects in BC, however, in case of SL this was not possible due to random effect variance equalling zero. Consequently, we used the following linear model for SL: ~ Temperature * F2 Diet + F1 Diet * F2 Diet. For BC we used the following linear mixed-effects model: ~ Temperature * F2 Diet + F1 Diet * F2 Diet + (1|Tank ID).

Maternal investment and reproductive success

To test for differences in clutch size of the P generation, while accounting for female size, we used a linear model including both origin and female SL as fixed effects. To test for determinants of clutch size in the F1 females, we included parental origin (CS vs. HS), temperature (cold vs. warm), diet (benthic vs. epibenthic) and their interactions as fixed effects. We also corrected for female body size, however, as F1 female body size itself is affected by rearing conditions, we used the residuals from the model testing for treatment (temperature and F1 diet) and parental origin effects on F1 female SL. Furthermore, we included family identity as a random effect to correct for family level effects. Thus, the final model for F1 clutch size was: ~ Temperature * F1 Diet + Temperature * Origin + F1 Diet * Origin + Female SL + (1|Family ID). Finally, for one F1 clutch within the warm-benthic-HS-treatment combination, all embryos died before reaching eye stage, indicating an egg quality issue. Thus, we removed this clutch from all subsequent analyses.

To test for differences in clutch mean egg size of F1 females, we applied linear mixed-effects model, including temperature (cold vs. warm), diet (benthic vs. epibenthic), parental origin (CS vs. HS) and all two-way interactions as fixed effects. Since we measured 20 eggs per female, we included female ID as a random effect to account for individual variation within families. Thus, the final model for Egg size was: ~ Temperature * F1 Diet + Temperature * Origin + F1 Diet * Origin + (1|Family ID/Female ID). We also ran a simple linear regression to test for the overall effect of clutch size on within clutch mean egg size.

To test for differences in fertilization and hatching success we used generalized linear models with a quasibinomial distribution, as we used the proportion of fertilized eggs per clutch and proportion of fertilized eggs that hatched per clutch. For the F1 generation, the model for fertilization success contained only P origin (CS vs. HS). For F1 hatching success, we further included incubation temperature (cold vs. warm) and the interaction between temperature and origin as fixed effects. To analyse fertilization and hatching success in the F2 generation, we included temperature (cold vs. warm), F1 diet (benthic vs. epibenthic), Origin (CS vs. HS) and their two-way interactions as fixed effects. Thus, the final model for fertilisation and hatching success was: ~ Temperature* F1 Diet + Temperature * Origin + F1 Diet * Origin.

3.4 Results

3.4.1 Analyses of juvenile and adult female body size

In the F1 generation, while average juvenile weight was lower in the warm treatment (Fig. 3.4A), juvenile weight was affected by an interaction between temperature and diet, while parental origin had no effect (Table 3.3, Fig. 3.4A). In the warm treatment, F1 juveniles reared on epibenthic diet were noticeably lighter than those reared on a benthic diet, while in the cold treatment, there was no difference in weight between the two diet groups (Table 3.3, Fig. 3.4A). In contrast, in the F2 generation juveniles reared in the cold treatment were clearly heavier than those reared in the warm treatment (Table 3.3, Fig. 3.4B), and within both temperature treatments F2 juveniles were heavier when reared on an epibenthic diet than benthic diet (Table 3.3, Fig. 3.4B). Furthermore, we found a positive effect of F1

epibenthic diet on F2 juvenile weight (Table 3.3). Finally, we also found a temperature-F2 diet interaction and a temperature-origin interaction, whereas origin on its own had no effect (Table 3.3).

There was no difference in standard length between the CS and HS origins of the P females (Table 3.3). However, P females from CS had a higher BC (Fulton's K)) than females from HS (Table 3.3). In F1 females, origin (CS or HS) had an effect on SL but no effect on F1 female BC (Table 3.3). For F1 females, there was a temperature-diet treatment interaction (Table 3.3). In the warm treatment, females reared on a benthic diet were larger than females reared on an epibenthic diet. In contrast, in the cold treatment, there was no difference in female SL between the two diet treatments (Fig. 3.5A). Likewise, there was a temperature-diet treatment interaction on body condition (Table 3.3): While F1 females fed the epibenthic diet tended to have a lower body condition in both temperature treatments, female condition was substantially lower in the cold-epibenthic diet treatment combination (Fig. 3.5B). For the F2 generation females, there was an effect of both temperature and F2 diet (Table 3.3). Females were, on average, shorter in the warm and in the epibenthic diet treatment (Fig. 3.5C). In contrast, there was no effect of temperature and diet on body condition in F2 females (Table 3.3, Fig. 3.5D).

3.4.2 Maternal investment and reproductive success

The wild collected P females from CS had larger clutches than HS females, even after correcting for female body length (Table 3.4). For F1 females, body length was positively correlated with clutch size (Table 3.4) and females produced on average larger clutches on a benthic diet compared to an epibenthic diet, and when reared in the warm compared to the cold (Table 3.4, Fig. 3.6A). However, we found no effect of origin on clutch size in F1 females (Table 3.4). For egg size, we found that F1 females reared in the cold or on an epibenthic diet had on average larger eggs compared to females reared in the warm and on a benthic diet (Table 3.4, Fig. 3.6B). Overall, clutch size of F1 females was negatively correlated with within clutch mean egg size, thus bigger clutches contained on average smaller eggs than smaller clutches ($F = 16.3$, $p < 0.001$, Fig. 3.7). For P females, there was no difference between origins in fertilization and hatching success of their F1 embryos (Table 3.4). In the F2 generation embryos, however, the warm temperature and CS origin strongly decreased fertilization and hatching success (Table 3.4; Fig. 3.3B)

Table 3.2 *Sample size overview of second generation threespine stickleback of a plasticity experiment with individuals reared in contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic (midge larvae) vs. epibenthic (Cladocera)). F2 individuals were reared in the same temperatures as their F1 parents, whereas F1 parental diet was either matched (benthic-benthic / epibenthic-epibenthic) or contrasted (benthic-epibenthic or epibenthic-benthic) (see Fig. 2). Cold Shore and Hot Shore refer to the grand-parental (P) habitat within Lake Mývatn (Fig. 1).*

Temperature	*F1 Diet*	*F2 Diet*	*Origin*	*Nr. crosses*	*F2 juveniles*	*F2 females*
Cold	**Benthic**	**Benthic**	**Cold Shore**	7	112	13
			Hot Shore	9	141	10
		Epibenthic	**Cold Shore**	7	123	16
			Hot Shore	9	192	12
	Epibenthic	**Benthic**	**Cold Shore**	9	103	10
			Hot Shore	7	70	10
		Epibenthic	**Cold Shore**	9	121	15
			Hot Shore	7	85	9
Warm	**Benthic**	**Benthic**	**Cold Shore**	10	65	12
			Hot Shore	4	24	2
		Epibenthic	**Cold Shore**	10	62	12
			Hot Shore	4	26	4
	Epibenthic	**Benthic**	**Cold Shore**	7	36	2
			Hot Shore	13	149	11
		Epibenthic	**Cold Shore**	7	32	3
			Hot Shore	13	192	17

Table 3.3 *Results of linear and linear mixed-effects models used to investigate the effects of temperature and diet on Threespine stickleback reared in a transgenerational experiment on contrasting temperatures (cold: 12°C vs. warm 22°C), and diets (benthic (midge larvae) vs. epibenthic (Cladocera)). Origin refers to parental and grandparental (P) origin within Lake Mývatn (Cold Shore and Hot Shore) (Fig. 1). Residual standard deviation, b and standard errors were extracted with the base R "summary" function* (R Core Team 2023). *Residual degrees of freedom, F and p-values were obtained with the "Anova" function (statistic = F and type = II) from the car package* (Fox and Weisberg 2018).

Focal trait	***N***	***Model type***	***Random effects***	***Fixed effects***	***b ± SE***	***Df res.***	***F***	***Type II p-value***
F1 juvenile weight	2153	lmer	Family/Tank identity: Residual SD = 0.028	**Temp.**	**-0.018 ± 0.009**	81.5	**117.12**	**< 0.001**
				F1 Diet	**0.004 ± 0.009**	79.1	**48.12**	**< 0.001**
				Origin	0.003 ± 0.011	70.0	0.13	0.721
				Temp. * F1 Diet	**-0.080 ± 0.011**	79.3	**56.75**	**< 0.001**
				Temp * Origin	-0.003 ± 0.010	105.8	0.07	0.789
				F1 Diet * Origin	0.003 ± 0.010	100.1	0.12	0.735
F2 juvenile weight	588	lmer	Tank identity Residual SD = 0.035	**Temp.**	**-0.048 ± 0.008**	107.6	**240.9**	**< 0.001**
				F1 Diet	**0.015 ± 0.006**	105.7	**4.3**	**0.041**
				F2 Diet	**0.066 ± 0.007**	104.4	**129.9**	**< 0.001**
				Origin	0.003 ± 0.006	105.3	1.3	0.254
				Temp. * F2 Diet	**-0.027 ± 0.009**	105.9	**9.1**	**0.003**
				Temp * Origin	**-0.022 ± 0.010**	108.6	**5.2**	**0.024**
				F1 Diet * F2 Diet	-0.004 ± 0.009	105.4	0.03	0.869

Table 3.3 *(continued)*

Focal trait	***N***	***Model type***	***Random effects***	***Fixed effects***	***b ± SE***	***Df res.***	***F***	***Type II p-value***
P female length	33	lm		Origin	-0.38 ± 1.63	31	-0.05	0.818
F1 female length	234	lmer	Family/Tank identity: Residual SD = 2.579	**Temperature**	**4.50 ± 0.77**	42.9	**43.95**	**< 0.001**
				F1 Diet	**-0.74 ± 0.75**	32.0	**11.35**	**0.002**
				Origin	1.28 ± 1.03	19.5	3.72	0.067
				Temp. * F1 Diet	**-1.96 ± 0.92**	33.1	**4.48**	**0.042**
				Temp. * Origin	-0.46 ± 1.01	38.2	0.20	0.658
				F1 Diet * Origin	0.57 ± 0.93	30.0	0.38	0.543
F2 female length	158	lm		**Temperature**	**-2.76 ± 0.67**	152	**24.55**	**< 0.001**
				F1 Diet	0.65 ± 0.65		0.02	0.889
				F2 Diet	**-2.49 ± 0.70**		**38.47**	**< 0.001**
				Temp. * F2 Diet	0.99 ± 0.90		1.21	0.273
				F1 Diet * F2 Diet	-1.27 ± 0.88		2.10	0.150
P female body condition	33	lm		**Origin**	**-0.179 ± 0.09**	31	**4.40**	**0.044**
F1 female body condition	234	lmer	Family/Tank identity: Residual SD = 0.106	**Temperature**	**-0.022 ± 0.030**	39.8	**4.38**	**0.043**
				F1 Diet	**-0.159 ± 0.029**	31.5	**44.64**	**< 0.001**
				Origin	0.004 ± 0.043	20.2	0.02	0.881
				Temp. * F1 Diet	**0.111 ± 0.035**	31.7	**9.80**	**0.004**
				Temp. * Origin	0.017 ± 0.040	34.2	0.18	0.677
				F1 Diet * Origin	-0.035 ± 0.035	28.4	0.97	0.333
F2 female body condition	158	lmer	Tank identity: Residual SD = 0.074	Temperature	0.019 ± 0.029	75.4	2.65	0.108
				F1 Diet	-0.031 ± 0.031	74.7	2.66	0.107
				F2 Diet	-0.030 ± 0.021	75.3	0.94	0.336
				Temp. * F2 Diet	0.024 ± 0.039	76.0	0.36	0.551
				F1 Diet * F2 Diet	0.007 ± 0.038	75.3	0.03	0.861

Table 3.4 *Results of linear, linear mixed-effects models and general linear models used to investigate the effects of temperature and diet on maternal investment and embryonic performance of F1 Threespine stickleback reared in a transgenerational experiment on contrasting temperatures (cold: 12°C vs. warm 22°C), and diets (benthic (midge larvae) vs. epibenthic (Cladocera)) (see Fig. 2). Origin refers to parental (P) origin within Lake Mývatn (Cold Shore and Hot Shore) (Fig. 1). Residual standard deviation, b and standard errors were extracted with the base R "summary" function (R Core Team, 2023). Residual degrees of freedom, F and p-values were obtained with the "Anova "function (statistic = F and type = II) from the car package (Fox and Weisberg, 2018).*

Focal trait	***N***	***Model type***	***Random effects***	***Fixed effects***	***b ± SE***	***Df res.***	***F***	***Type II p-value***
P clutch size	33	lm		**Origin**	**-49.94 ± 15.92**	30	**3.14**	**0.004**
				Fem. body length	**6.21 ± 1.75**		**3.54**	**0.001**
F1 Clutch size	83	lmer df = 78	Family: Residual SD = 22.45	**Temp.**	**23.43 ± 8.24**	64.9	**12.53**	**< 0.001**
				F1 Diet	**-33.11 ± 8.37**	73.0	**33.13**	**< 0.001**
				Origin	-2.08 ± 10.02	15.8	0.70	0.417
				Res. Fem. length	**4.22 ± 1.15**	72.4	**13.03**	**< 0.001**
				Temp. * F1 Diet	-2.42 ± 9.70	70.8	0.06	0.805
				Temp * Origin	-7.60 ± 10.30	63.6	0.50	0.480
				F1 Diet * Origin	12.73 ± 9.79	72.6	1.64	0.205
F1 Egg size	1600	lmer	Family/Mother ID: Residual SD = 0.052	**Temp.**	**-0.144 ± 0.024**	71.46	**86.14**	**< 0.001**
				F1 Diet	**0.041 ± 0.024**	69.95	**12.98**	**< 0.001**
				Origin	0.046 ± 0.030	13.40	0.00	0.962
				Temp. * F1 Diet	0.049 ± 0.029	70.53	2.87	0.094
				Temp * Origin	**-0.059 ± 0.031**	69.01	**3.45**	**0.066**
				F1 Diet * Origin	-0.025 ± 0.029	70.01	0.72	0.400

Table 3.4 *(continued)*

Focal trait	***N***	***Model type***	***Random effects***	***Fixed effects***	***b ± SE***	***Df res.***	***F***	***Type II p-value***
F1 Fertilization success	33	glm		Origin	0.24 ± 0.26	64	0.87	0.353
F2 Fertilization success	83	glm		**Temperature**	**-4.62 ± 1.79**	76	**100.44**	**< 0.001**
				F1 Diet	1.90 ± 2.72		0.03	0.857
				Origin	**-0.25 ± 2.21**		**4.12**	**0.046**
				Temp. * F1 Diet	-1.72 ± 2.72		0.51	0.476
				Temp * Origin	1.24 ± 2.24		0.32	0.576
				F1 * Diet	-0.70 ± 0.58		1.47	0.229
F1 Hatching success	33	glm		Temperature	0.26 ± 0.33	62	1.93	0.170
				Origin	-0.33 ± 0.32		1.44	0.235
				Temp. * Origin	0.11 ± 0.46		0.06	0.814
F2 Hatching success	82	glm		**Temperature**	**-2.89 ± 0.60**	75	**56.00**	**< 0.001**
				F1 Diet	-0.09 ± 0.64		1.38	0.245
				Origin	**-0.53 ± 0.64**		**4.08**	**0.047**
				Temp * F1 Diet	0.19 ± 0.68		0.08	0.781
				Temp. * Origin	1.15 ± 0.68		2.82	0.097
				F1 Diet * Origin	0.69 ± 0.64		1.18	0.281

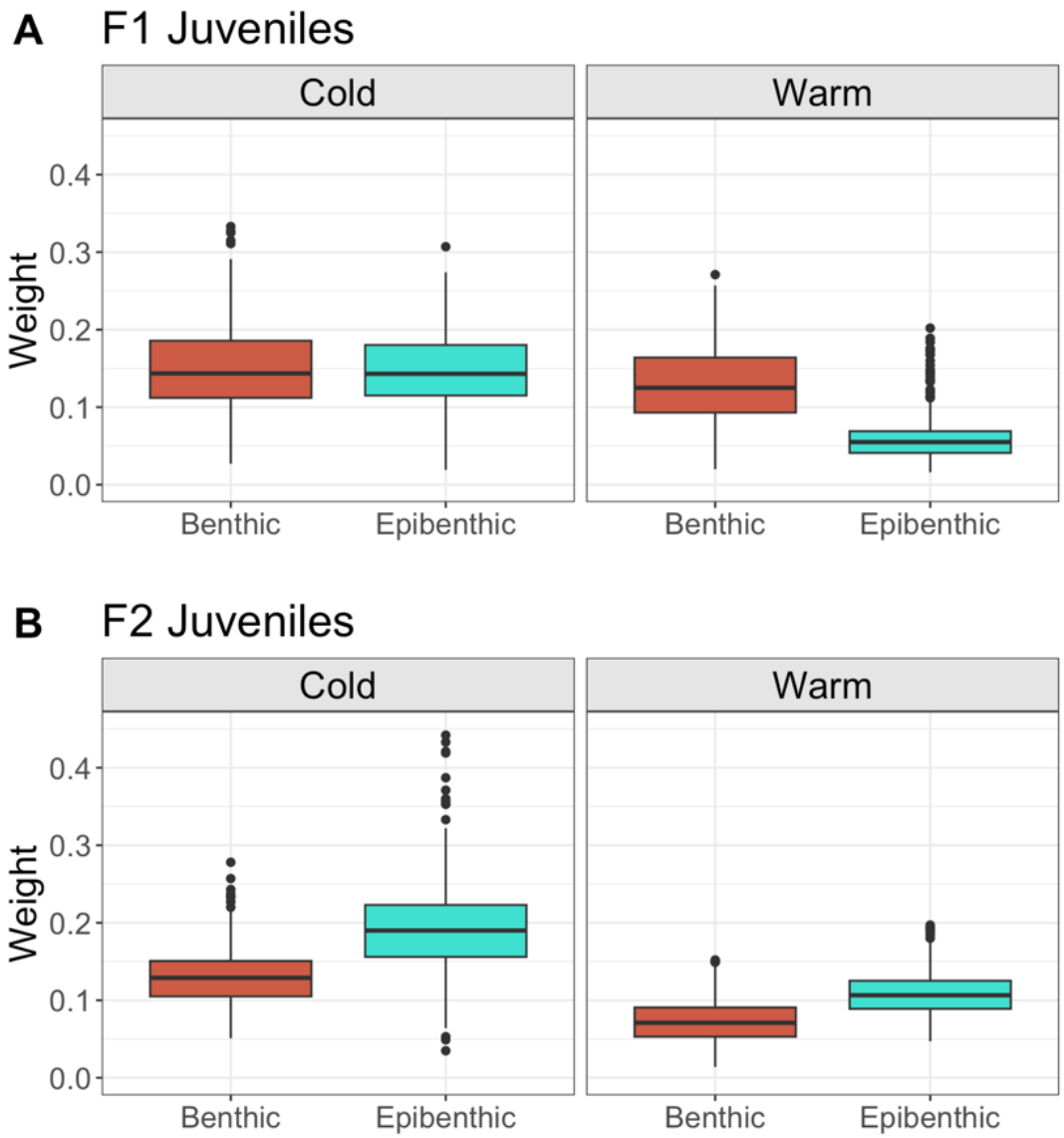

Figure 3.4 *Weight (g) of juvenile threespine stickleback reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic (midge larvae) vs. epibenthic (Cladocera)) across two generations (see Fig. 2). Points represent uncorrected measurements of individual juveniles. A) Weight (in g) of individuals of the F1 generation in the plasticity experiment. B) Weight of individuals of the F2 generation in the plasticity experiment. F2 juveniles were reared in the same temperatures as their F1 parents, whereas F1 parental diet was either matched (benthic-benthic / epibenthic-epibenthic) or contrasted (benthic-epibenthic or epibenthic-benthic) in the F2 generation (see Fig. 2). For F2 juveniles we only show effects of F2 diets as there was no significant interaction between F1 and F2 diet. Boxplots show the median (horizontal line), interquartile range (box), and 1.5× interquartile range whiskers; each dot represent an individual outside this range (outliers).*

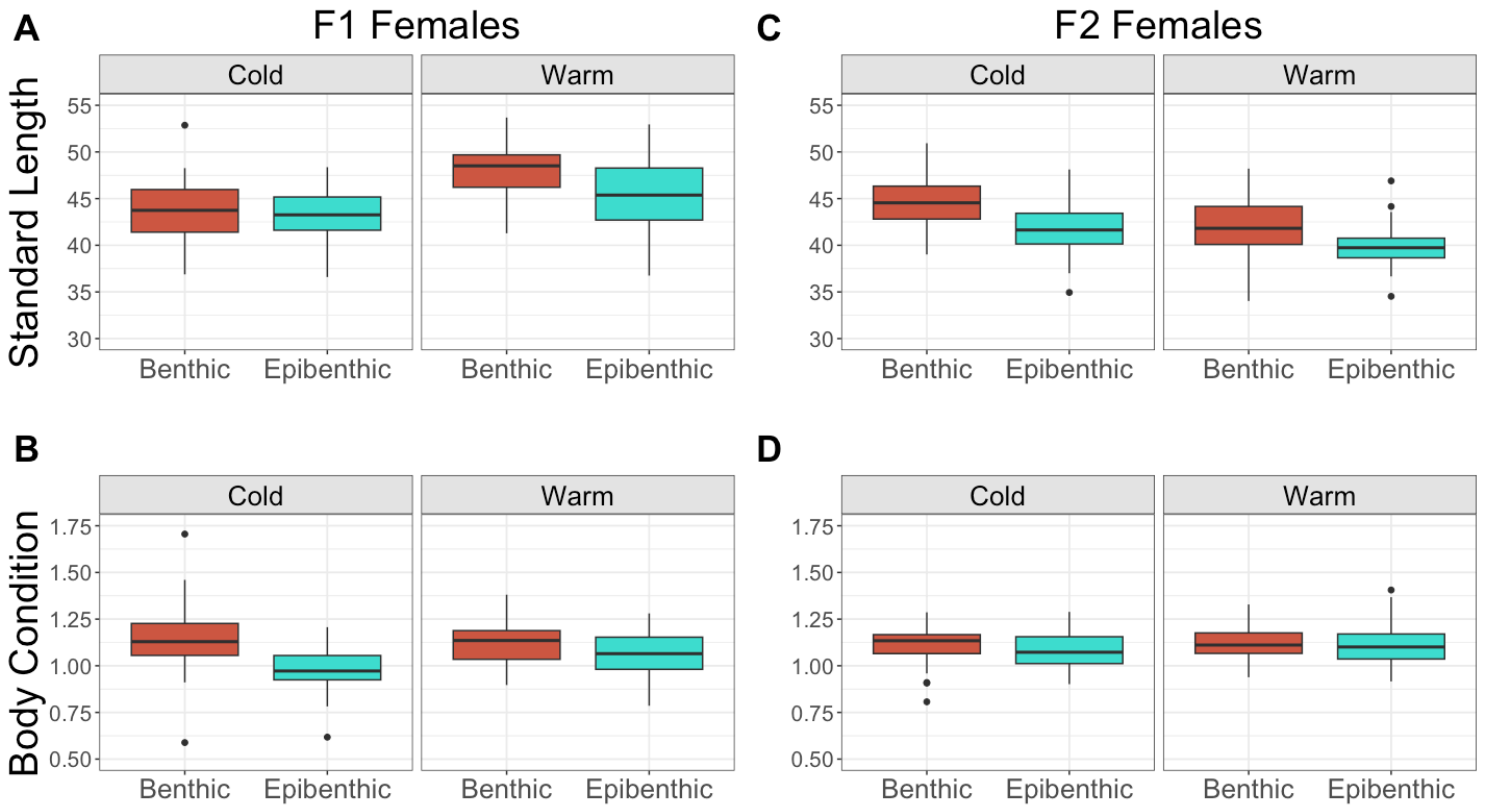

Figure 3.5 *Standard body length (mm) and body condition (Fulton's K) of sexually mature female threespine stickleback reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic (midge larvae) vs. epibenthic (Cladocera)) (see Fig. 2). Individual points represent uncorrected measurements of standard body length (mm) and body condition (Fulton's K,* (Nash et al. 2006)*) of individual females. A&B F1 females reared in combination of contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic vs. epibenthic). C&D F2 females reared in the same temperatures as their F1 parents, whereas F1 parental diet was either matched (benthic-benthic / epibenthic-epibenthic) or contrasted (benthic-epibenthic or epibenthic-benthic) in the F2 generation (see Fig. 2). For F2 females we only show effects of F2 diets as there was no significant interaction between F1 and F2 diet. Boxplots show the median (horizontal line), interquartile range (box), and 1.5× interquartile range whiskers; each dot represent an individual outside this range (outliers).*

3.5 Discussion

The main aim of this study was to test the effects of temperature and diet induced phenotypic plasticity within and across generations on key life history traits components (growth and reproduction) of stickleback, and for this we used juvenile and adult threespine stickleback originating from cold (Cold Shore, CS) versus warm (Hot Shore, HS) habitats in Lake Mývatn. We found that while environmental conditions played a pivotal role in shaping size, the effects depended on the life stage. In terms of temperature, warm (22°C) rearing conditions reduced juvenile size (weight) relative to cold (12°C) rearing conditions but resulted in increased female size (length) at reproduction. Furthermore, while warm rearing conditions reduced hatching success of the F2 generation and therefore fitness of their parents, offspring of HS individuals had higher hatching success at warm temperatures. Consequently, F1 individuals with a HS origin had a fitness advantage, indicating temperature induced adaptive divergence or transgenerational effects. Diet of the F1 generation influenced maternal investment, where F1 females reared on an epibenthic diet produced bigger but fewer eggs. Moreover, this increase in egg size was associated with increased size of juveniles, as well as adult females. As survival of juvenile (Reimchen 1990) and reproductive success of females (Wootton 1977; Baker et al. 2015; Kasimatis and Riginos 2016) are highly correlated with body size in stickleback, our results indicate that egg size-mediated transgenerational effects can have fitness consequences. Our results suggest that threespine stickleback can modulate key life-history components through adaptive within and across generation phenotypic plasticity, which may help them cope with adverse environmental conditions.

3.5.1 Phenotypic plasticity in growth and reproduction

Threespine stickleback are known for their high degree of adaptive potential through high standing genetic variation and capacity for phenotypic plasticity, which has allowed them to rapidly adapt to various environments (Schluter 1996; Nosil 2012). Especially contrasting environmental conditions, such as temperature, diet and salinity, during development have been reported to elicit plastic responses in body shape and size, trophic morphology (e.g. gill raker number and length), egg size and survival (Day et al. 1994; Ramler et al. 2014; Delarue 2016; Heckwolf et al. 2018). Furthermore, juvenile survival is strongly impacted by size in stickleback (Reimchen 1990) and therefore, early growth may be tightly linked to fitness. Consequently, we expected a high degree of plasticity in juvenile body size in response to temperature and diet.

In terms of temperature induced variation, juveniles in the F1 generation were, on average, smaller when reared in warm temperatures and on an epibenthic diet. Whereas, in the F2 generation, juveniles were bigger in the cold and on an epibenthic diet. This is in line with individuals growing faster in colder temperatures due to lower energetic requirements, as seen in numerous previous studies (Sibly and Atkinson 1994; Pörtner et al. 2001; Angilletta et al. 2004; Ramler et al. 2014). However, we found that in warm rearing conditions F1 females were larger at maturity than in cold rearing conditions. This contrasts with studies that find that towards the thermal maximum fish growth is diminished, and maturation occurs at a smaller size due to faster development (Pörtner et al. 2001; Kuparinen et al. 2011; Donelson et al. 2014). However, while previous studies have generally contrasted optimal rearing temperatures with elevated temperatures, we reared the fish cold (12°C) and warm (22°C), which represent the two thermal extremes observed in the wild.

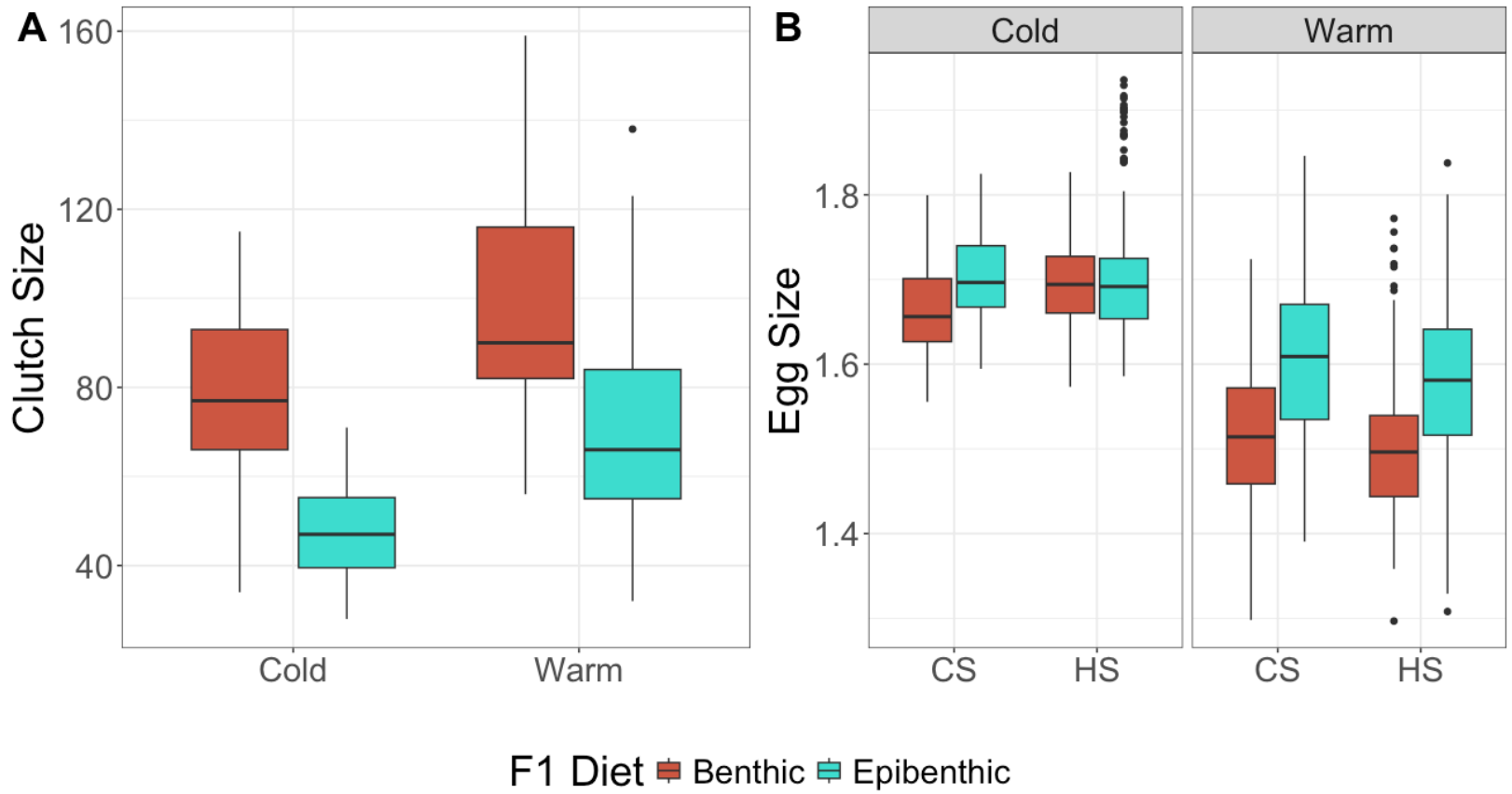

Figure 3.6 *Clutch size and eggs size of F1 female threespine stickleback reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic (midge larvae) vs. epibenthic (Cladocera)) (see Fig. 2). A) Clutch size (total number of eggs per clutch) of F1 females reared in the plasticity experiment. Points represent individual clutches. B) Egg size (mean of four diameters per egg in mm) of clutches collected from F1 females reared in the plasticity experiment. The x-axis indicates grandparental origin within Lake Mývatn; Cold Shore (CS) and Hot Shore (HS) (Fig. 1). Points represent measurements of individual eggs. Boxplots show the median (horizontal line), interquartile range (box), and 1.5× interquartile range whiskers; each dot represent A) individual clutches or B) individual eggs outside this range (outliers).*

Interestingly, our results align with findings on Japanese medaka (*Oryzias latipes*) that were reared 5°C above and below the thermal optimum (25°C), which found that fish reared in warm treatment conditions across multiple generations were larger at maturation than fish reared below optimal rearing temperatures (Loisel et al. 2019). Indeed, suboptimal low environmental temperature can cause serious challenges in fish, such as cold-induced fasting, metabolic depression, and lower immune capacity (Ibarz et al. 2010). Consequently, growth and reproduction can be energetically challenging in cold conditions. An alternative explanation for our cold reared F1 females to be smaller than their warm reared counterparts could be that the fish in our experiment were not energetically limited due to the *ad libitum* feeding and supplementing with food pellets high in nutrients and energy, which may have allowed females to maximise their growth even under energetically more demanding warm rearing temperatures. This appears a more plausible explanation, as Mývatn stickleback are generally smaller in the warm environment (HS) compared to stickleback in other parts of the lake (Millet et al. 2013; Strickland et al. 2023). Additionally, we found no effect of origin in our experiment, which might otherwise suggest a genetic basis for smaller body size in HS.

In terms of diet induced variation, we found a difference between the two diet treatments (benthic vs. epibenthic) in size at juvenile and adult life stages, as well as maternal investment. This is especially interesting, when considering that both our diet treatments were supplemented with aquaculture pellets, that are high in nutritional and energetic value. Nevertheless, our results suggest that an epibenthic diet is less energetically rewarding because adult F1 females show a lower body condition across both temperature treatments.

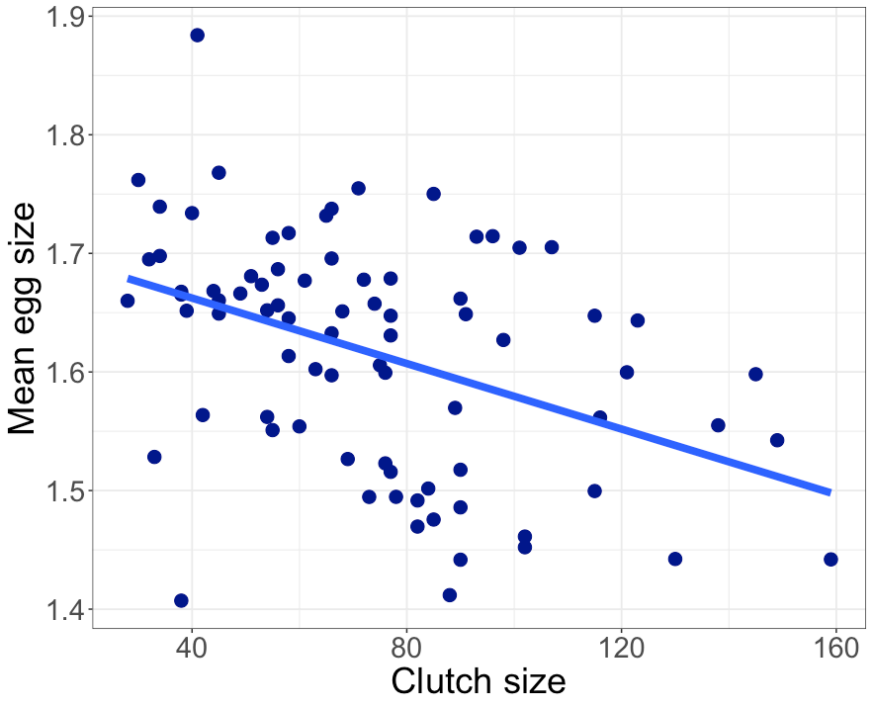

Figure 3.7 *Mean egg size (mm) and clutch size of F1 female threespine stickleback reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic vs. epibenthic) (see Fig. 2). Points represent uncorrected measurements of clutch size (total number of eggs per clutch) and mean egg size per clutch (mm) of individual clutches. The line is a simple linear regression generated with the "geom_smooth" function from the ggplot2 package* (Wickham 2016).

Indeed, Cladocera, which largely make up the epibenthic diet, have a more calcified shell which may be more challenging to digest. Cladocera are also mobile prey in the water column which requires more agile foraging strategy of a relatively small prey. In comparison, midge larvae are overall bigger and less mobile. This may decrease the net energy gain obtained from the same volume of Cladocera compared to midge larva. Moreover, multiple studies on wild juvenile threespine stickleback show that they transition to a more midge-based diet with increasing body size (Demchuk et al. 2015), further suggesting that midge larvae are energetically a more favourable diet compared to Cladocera (Delbeek and Williams 1988; Hangelin and Vuorinen 1988; Demchuk et al. 2015). On the other hand, Cladocera are better quality food than midges terms of their essential fatty acid composition (Caf et al. 2020; Chakraborty and Mallick 2023), which is needed for optimal growth and reproduction. However, to ensure that both diet treatments contained all necessary nutrients, we supplemented both diets with aquaculture pellets.

3.5.2 Maternal investment and transgenerational effects

According to life history theory, individuals optimise energy allocation between growth and reproduction on the one hand, and between offspring size and number on the other, to maximise lifetime fitness (Stearns 1998; Kaplan and Robson 2009). In many oviparous species with indetermined growth, size is a good predictor for female fecundity (Wootton 1977; Baker et al. 2015; Kasimatis and Riginos 2016). Under resource limited conditions females may face a trade-off between allocating energy to growth versus reproduction, and reduced growth rate may result in postponing maturation or reproduction (Baker et al. 2008). However, the amount of energy a female can invest into reproduction is also dependent on environmental conditions and energy reserves, as illustrated by a laboratory study showing an increased likelihood of females developing a clutch with increasing female body condition in threespine stickleback (Bagamian et al. 2004). Furthermore, maternal investment to egg number *versus* egg size and composition, can be environment dependent and vary within and among females and, consequently, result in variation in offspring quality (Räsänen and Kruuk 2007; Moore et al. 2019; Marks and Lailvaux 2024).

In threespine stickleback, body size is the most consistent predictor of clutch size, and clutch size is commonly negatively correlated with egg size (Wootton 1973; Baker et al. 2015). Our results are in line with these previous observations as we found that F1 female size and clutch size were positively correlated, whereas egg size and clutch size were negatively correlated. We also observed that females reared in cold conditions produced larger eggs, likely in response to longer incubation time in cold temperatures, which is congruent with previous studies (Einum et al. 2002; Leblanc et al. 2016). Moreover, we observed a drastic reduction in fertilization and hatching success (key components of reproductive success) in warm rearing conditions. According to life history theory, such elevated offspring mortality may favour the production of many small offspring (Ernsting and Isaaks 2000). In line with this, we observed that warm reared females had more, but smaller eggs compared to cold reared females. However, we observed that clutches of females reared in warm conditions had higher fertilization success if their (grand)parents originated from warm habitats (HS). Thus, either genetic or transgenerational effects may have increased fertility of F1 females.

We also found that females altered their maternal investment based on their diet, and that this effect was stronger in the cold rearing environment. Specifically, F1 females reared on an epibenthic diet, invested in fewer but larger eggs compared to females reared on a benthic diet. As females reared on an epibenthic diet also had a lower body condition, body condition appears to be an important determinant for female reproductive investment. In a study that compared egg size of wild reared rainbow trout females (*Oncorhynchus mykiss*) to first generation aquaculture females (meaning wild caught parents), which were reared on a high-quality diet for one year,

before being released into the wild as smolts, wild collected females produced fewer but larger eggs, compared to the first generation aquaculture females (Leblanc et al. 2023). In threespine stickleback, few studies report no change in body condition in response to different diets or parasite infestation in wild caught females (Bagamian et al. 2004; Pawelec et al. 2016). However, more studies are necessary to draw any definitive conclusions of the effect of body condition on female fertility.

The variation in material investment of the F1 females in our experiment had long lasting effects into the next generation. First, cold-reared F2 juveniles were larger if their F1 parents had been reared on an epibenthic diet, and these F1 females also produced bigger eggs. It was clear that across all treatment combinations, larger eggs resulted in larger offspring at juvenile and adult life stages. Previous studies have reported that transgenerational effects in response to environmental stressors, such as rising temperatures, have the potential to compensate for deficits in offspring traits caused by earlier unfavourable conditions (Donelson et al., 2011; Miller et al., 2012; Salinas and Munch, 2012). However, other studies suggest that complete compensation does not always occur (Shama et al., 2014a, 2016; Metcalfe, 2024). In our experiment, we found clear transgenerational effects on hatching success and juvenile and adult size, whereby a reduction in clutch size (egg number) in favour of increased eggs size could mitigate some of the negative environmental effects, which could indicate adaptive transgenerational effects. Our results support findings of previous studies, that transgenerational effects can be important in mitigating adverse environmental conditions (Räsänen et al. 2005; Shama et al. 2014; Shama et al. 2016; Metcalfe 2024).

All our observations taken together make a strong case for elevated rearing temperatures being a challenging environment for Mývatn threespine stickleback, especially reducing female condition, fecundity, and early life performance, hence directly impacting key fitness components. However, it is possible that transgenerational effects can compensate for reduced maternal condition in challenging environmental conditions. Furthermore, we find parental or grandparental effects to be especially influential at embryonic life stage when selection pressures generally are strong. Moreover, after two generations we observe similar patterns of phenotypic divergence in our experiments as we do in the wild for the Mývatn stickleback: larger body size in cold habitats and on a benthic diet (Millet 2013; Snorradóttir 2023; Strickland et al. 2023). Our results strengthen our understanding of how life history plasticity might facilitate persistence of stickleback in the thermally challenging environment of the HS environment.

Finally, diet and temperature can cause interactive effects on phenotypic variation, which highlights the importance of testing phenotypes in more ecologically relevant and complex contexts. Due to integrating two major environmental drivers (temperature and diet) of phenotypic variation in this threespine stickleback population, we gained insight to the role of phenotypic plasticity in phenotypic divergence in face of gene flow and absence of strong genetic divergence (Strickland et al. 2023). This suggests that within and across generation plasticity may be favoured over local adaptation in this population subjected to high spatial and temporal variation in the wild.

3.5.3 Conclusions

Results from our transgenerational experiment provide evidence for how different environmental factors (here temperature and diet) can interact and affect phenotypic variation via plasticity. Specifically, the contrasting temperature and diet treatments elicited variation in key life history traits within and across generations, directly influencing fitness components. Furthermore, the results from this study reinforce that the effects of environmental factors can vary across different life stages and generations as the effects of a given environmental manipulation differed

between juveniles vs. adults as well as between F1 and F2 generations. Moreover, we showed that both low and elevated temperatures can pose challenges for organismal performance, but that, especially in warm rearing conditions, transgenerational effects may alleviate some of the adverse fitness implications. To understand how environmental conditions may affect fitness components at different developmental stages and shape phenotypic variation, running experiments across generations is crucial. Finally, our study aids in our understanding how phenotypic plasticity and transgenerational effects can be favoured in individuals inhabiting highly dynamic systems.

Acknowledgments

Mývatn is a protected area, thus, all sampling was done in collaboration with RAMÝ (Mývatn nature research station), who holds all permissions to sample in lake Mývatn. Further, permissions from local landowners were obtained to conduct this study on their properties. This study was supported by the Icelandic Research Fund, grant of excellence (195571-052) and doctoral student grant (228501-051). Moreover, we would like to thank all students and technicians that were instrumental in conducting this experiment. We especially thank Stephen Price who developed and maintained the Cladocera rearing system and assisted with daily management of the experiment as well as developing sampling protocols. Finally, we thank Kári H. Árnason and Camille A. Leblanc for their invaluable support throughout this project.

References

Angilletta MJ Jr, Steury TD, Sears MW. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol. 44(6):498–509.

Bagamian KH, Heins DC, Baker JA. 2004. Body condition and reproductive capacity of three-spined stickleback infected with the cestode *Schistocephalus solidus*. J Fish Biol. 64(6):1568–1576.

Baken EK, Collyer ML, Kaliontzopoulou A, Adams DC. 2021. geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. Methods Ecol Evol. 12(12):2355–2363.

Baker JA, Heins DC, Foster SA, King RW. 2008. An Overview of Life-History Variation in Female Threespine Stickleback. Behaviour. 145(4/5):579–602.

Baker JA, Wund MA, Heins DC, King RW, Reyes ML, Foster SA. 2015. Life-history plasticity in female threespine stickleback. Heredity . 115(4):322–334.

Bartrons M, Einarsson Á, Nobre RLG, Herren CM, Webert KC, Brucet S, Ólafsdóttir SR, Ives AR. 2015. Spatial patterns reveal strong abiotic and biotic drivers of zooplankton community composition in Lake Mývatn, Iceland. Ecosphere. 6(6):art105.

Bates D, Machler M, Bolker B, Walker SC. 2014. Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 67:1–48.

BioMar SAS. BioMar. [accessed 2024 Feb 20]. https://www.biomar.com/en-gb.

Bolnick DI, Ballare KM. 2020. Resource diversity promotes among-individual diet variation, but not genomic diversity, in lake stickleback. Ecol Lett. 23(3):495–505.

Boulé V, Fitzgerald GJ. 1989. Effects of constant and fluctuating temperatures on egg production in the threespine stickleback (*Gasterosteus aculeatus*). Can J Zool. 67(6):1599–1602.

Caf F, Özdemir N, Gökçe Z. 2020. Biochemical Content (fatty acids, sterols, total protein, lipophilic vitamins) and Antioxidant activity (GSH, MDA) of Cladocerans (*Daphnia sp.*, *Diaphasoma branchyrum*). Medit Fish Aquac Res. 3:34–46.

Candolin U. 1999. The relationship between signal quality and physical condition: is sexual signalling honest in the three-spined stickleback? Anim Behav. 58(6):1261–1267.

Carney Almroth B, Asker N, Wassmur B, Rosengren M, Jutfelt F, Gräns A, Sundell K, Axelsson M, Sturve J. 2015. Warmer water temperature results in oxidative damage in an Antarctic fish, the bald notothen. J Exp Mar Bio Ecol. 468:130–137.

Chakraborty S, Mallick PH. 2023. Cladocera as a substitute for Artemiaas live feed in aquaculture practices: a review. Sust Agric Food Env Res. 11.

Cresko WA, McGuigan KL, Phillips PC, Postlethwait JH. 2007. Studies of threespine stickleback developmental evolution: progress and promise. Genetica. 129(1):105–126.

Crichigno SA, Battini MÁ, Cussac VE. 2014. Diet induces phenotypic plasticity of *Percichthys trucha* (Valenciennes, 1833) (Perciformes, Percichthyidae) in Patagonia. Zool Anz. 253(3):192–202.

Currey MC, Bassham S, Perry S, Cresko WA. 2017. Developmental timing differences underlie armor loss across threespine stickleback populations. Evol Dev. 19(6):231–243.

Danchin É, Charmantier A, Champagne FA, Mesoudi A, Pujol B, Blanchet S. 2011. Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. Nat Rev Genet. 12(7):475–486.

Day T, Pritchard J, Schluter D. 1994. A comparison of two sticklebacks. Evolution. 48(5):1723–1734.

Del Giudice M. 2015. Plasticity as a developing trait: exploring the implications. Front Zool. 12 Suppl 1(Suppl 1):S4.

Delarue C. 2016. Plasticity of trait divergence in threespine stickleback (*Gasterosteus aculeatus*) from Lake Mývatn. Kristjánsson B, Räsänen K, editors. MSc Thesis, Hólar University.

Delbeek JC, Williams DD. 1988. Feeding selectivity of four species of sympatric stickleback in brackish-water habitats in eastern Canada. J Fish Biol. 32(1):41–62.

Demchuk A, Ivanov M, Ivanova T, Polyakova N, Mas-Martí E, Lajus D. 2015. Feeding patterns in seagrass beds of three-spined stickleback *Gasterosteus aculeatus* juveniles at different growth stages. J Mar Biol Assoc UK. 95(8):1635–1643.

Donelson JM, McCormick MI, Booth DJ, Munday PL. 2014. Reproductive acclimation to increased water temperature in a tropical reef fish. PLoS One. 9(5):e97223.

Einarsson Á, Örnólfsdóttir EB. 2004. Long-term changes in benthic Cladocera populations in Lake Myvatn, Iceland. Aquat Ecol. 38(2):253–262.

Einarsson Á, Stefánsdóttir G, Jóhannesson H, Ólafsson JS, Már Gíslason G, Wakana I, Gudbergsson G, Gardarsson A. 2004. The ecology of Lake Myvatn and the River Laxá: Variation in space and time. Aquat Ecol. 38(2):317–348.

Einum S, Hendry AP, Fleming IA. 2002. Egg-size evolution in aquatic environments: does oxygen availability constrain size? Proc Biol Sci. 269(1507):2325–2330.

Ernsting G, Isaaks A. 2000. Ectotherms, Temperature, and Trade-offs: Size and Number of Eggs in a Carabid Beetle. Am Nat. 155(6):804–813.

Fawcett TW, Frankenhuis WE. 2015. Adaptive explanations for sensitive windows in development. Front Zool. 12 Suppl 1(Suppl 1):S3.

Fox J, Weisberg S. 2018. An R companion to applied regression. SAGE Publications.

Georga I, Koumoundouros G. 2010. Thermally induced plasticity of body shape in adult zebrafish *Danio rerio* (Hamilton, 1822). J Morphol. 271(11):1319–1327.

Gunter HM, Fan S, Xiong F, Franchini P, Fruciano C, Meyer A. 2013. Shaping development through mechanical strain: the transcriptional basis of diet-induced phenotypic plasticity in a cichlid fish. Mol Ecol. 22(17):4516–4531.

Hangelin C, Vuorinen I. 1988. Food selection in juvenile three-spined sticklebacks studied in relation to size, abundance and biomass of prey. Hydrobiologia. 157(2):169–177.

Heckwolf MJ, Meyer BS, Döring T, Eizaguirre C, Reusch TBH. 2018. Transgenerational plasticity and selection shape the adaptive potential of sticklebacks to salinity change. Evol Appl. 11(10):1873–1885.

Hegrenes S. 2001. Diet-induced phenotypic plasticity of feeding morphology in the orangespotted sunfish, *Lepomis humilis*. Ecol Freshw Fish. 10(1):35–42.

Houle D, Govindaraju DR, Omholt S. 2010. Phenomics: the next challenge. Nat Rev Genet. 11(12):855–866.

Ibarz A, Padrós F, Gallardo MÁ, Fernández-Borràs J, Blasco J, Tort L. 2010. Low-temperature challenges to gilthead sea bream culture: review of cold-induced alterations and 'Winter Syndrome.' Rev Fish Biol Fish. 20(4):539–556.

Ives AR, Einarsson Á, Jansen VAA, Gardarsson A. 2008. High-amplitude fluctuations and alternative dynamical states of midges in Lake Myvatn. Nature. 452(7183):84–87.

Johansson MP, Ermold F, Kristjánsson BK, Laurila A. 2016. Divergence of gastropod life history in contrasting thermal environments in a geothermal lake. J Evol Biol. 29(10):2043–2053.

Jonsson B, Jonsson N. 2014. Early environment influences later performance in fishes. J Fish Biol. 85(2):151–188.

Kaplan HS, Robson AJ. 2009. We age because we grow. Proc Biol Sci. 276(1663):1837–1844.

Kasimatis K, Riginos C. 2016. A phylogenetic analysis of egg size, clutch size, spawning mode, adult body size, and latitude in reef fishes. Coral Reefs. 35(2):387–397.

Kristjánsson BK, Skúlason S, Noakes DL. 2002. Morphological segregation of Icelandic threespine stickleback (*Gasterosteus aculeatus L.*). Biol J Linn Soc Lond. 76(2):247–257.

Kuparinen A, Cano JM, Loehr J, Herczeg G, Gonda A, Merilä J. 2011. Fish age at maturation is influenced by temperature independently of growth. Oecologia. 167(2):435–443.

Lahnsteiner F. 2011. Spermatozoa of the teleost fish *Perca fluviatilis* (perch) have the ability to swim for more than two hours in saline solutions. Aquaculture. 314(1):221–224.

Leblanc CA, Schreck C, Kristjánsson BK, Skúlason S, Noakes DLG. 2023. Egg size–related traits during the first year of growth and smolting in hatchery and wild juveniles of steelhead trout *Oncorhynchus mykiss*. Environ Biol Fishes. 106(5):1061–1078.

Leblanc CA-L, Kristjánsson BK, Skúlason S. 2016. The importance of egg size and egg energy density for early size patterns and performance of Arctic charr *Salvelinus alpinus*. Aquac Res. 47(4):1100–1111.

Levis NA, de la Serna Buzón S, Pfennig DW. 2015. An inducible offense: carnivore morph tadpoles induced by tadpole carnivory. Ecol Evol. 5(7):1405–1411.

Loisel A, Isla A, Daufresne M. 2019. Variation of thermal plasticity in growth and reproduction patterns: Importance of ancestral and developmental temperatures. J Therm Biol. 84:460–468.

Marks JR, Lailvaux SP. 2024. The Integrative Life history of Maternal Effects. Integr Comp Biol. 64(6):1623–1632.

McGee MD, Schluter D, Wainwright PC. 2013. Functional basis of ecological divergence in sympatric stickleback. BMC Evol Biol. 13:277.

Metcalfe NB. 2024. How important is hidden phenotypic plasticity arising from alternative but converging developmental trajectories, and what limits it? J Exp Biol. 227(Suppl_1).

Meyer A. 1987. Phenotypic plasticity and heterochrony in *Cichlasoma managuense* (Pisces, Cichlidae) and their implications for speciation in cichlid fishes. Evolution. 41(6):1357–1369.

Millet A. 2013. Spatio-temporal variation of threespine stickleback (*Gasterosteus aculeatus*) in the dynamic Lake Mývatn, Iceland. Kristjánsson BK, Räsänen K, editors. PhD thesis, University of Iceland.

Millet A, Kristjánsson BK, Einarsson Á, Räsänen K. 2013. Spatial phenotypic and genetic structure of threespine stickleback (*Gasterosteus aculeatus*) in a heterogeneous natural system, Lake Mývatn, Iceland. Ecol Evol. 3(10):3219–3232.

Moore MP, Whiteman HH, Martin RA. 2019. A mother's legacy: the strength of maternal effects in animal populations. Ecol Lett. 22(10):1620–1628.

Mousseau TA, Fox CW. 1998a. The adaptive significance of maternal effects. Trends Ecol Evol. 13(10):403–407.

Mousseau TA, Fox CW. 1998b. Maternal effects as adaptations. Oxford University Press.

Nash RDM, Valencia AH, Geffen AJ. 2006. The origin of Fulton's condition factor—setting the record straight. Fisheries. 31(5):236–238.

Nosil P. 2012. Ecological Speciation. Oxford University Press.

Ólafsdóttir GÁ, Snorrason SS, Ritchie MG. 2007. Postglacial intra-lacustrine divergence of Icelandic threespine stickleback morphs in three neovolcanic lakes. J Evol Biol. 20(5):1870–1881.

Ólafsson J. 1979. Physical Characteristics of Lake Mývatn and River Laxá. Oikos. 32(1/2):38–66.

Pawelec AJ, Sapota MR, Skóra ME. 2016. Is the body condition of the three-spined stickleback (*Gasterosteus aculeatus*) determined by the type of food consumed? Oceanol Hydrobiol Stud. 45(4):588–599.

Pfennig DW. 2021. Phenotypic Plasticity & Evolution: causes, consequences, controversies. Taylor & Francis (Evolutionary Cell Biology).

Pigliucci M. 2005. Evolution of phenotypic plasticity: where are we going now? Trends Ecol Evol. 20(9):481–486.

Pörtner H-O, Berdal B, Blust R, Brix O, Colosimo A, De Wachter B, Giuliani A, Johansen T, Fischer T, Knust R, et al. 2001. Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). Cont Shelf Res. 21(18–19):1975–1997.

Posit team. 2023. RStudio: Integrated Development for R. http://www.posit.co/.

Potts LB, Mandrak NE, Chapman LJ. 2021. Coping with climate change: Phenotypic plasticity in an imperilled freshwater fish in response to elevated water temperature. Aquat Conserv. 31(10):2726–2736.

Priborsky J, Velisek J. 2018. A Review of Three Commonly Used Fish Anesthetics. Reviews in Fisheries Science & Aquaculture. 26(4):417–442.

R Core Team. 2023. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Ramler D, Mitteroecker P, Shama LNS, Wegner KM, Ahnelt H. 2014. Nonlinear effects of temperature on body form and developmental canalization in the threespine stickleback. J Evol Biol. 27(3):497–507.

Räsänen K, Kruuk LEB. 2007. Maternal effects and evolution at ecological time-scales. Funct Ecol. 21(3):408–421.

Räsänen K, Laurila A, Merilä J. 2005. Maternal investment in egg size: environment- and population-specific effects on offspring performance. Oecologia. 142(4):546–553.

Rasband, W.S. , U.S. National Institutes of Health, Bethesda, Maryland, USA. 1997-2018. ImageJ. https://imagej.net/ij/.

Reimchen TE. 1990. Size-Structured Morality in a Threespine Stickleback (*Gastrosteus aculeatus*) – Cutthroat Trout (*Oncorhynchus clarki*) Community. Can J Fish Aquat Sci. 47(6):1194–1205.

Ritchie DJ, Friesen CR. 2022. Invited review: Thermal effects on oxidative stress in vertebrate ectotherms. Comp Biochem Physiol A Mol Integr Physiol. 263(111082):111082.

Roff D. 1994. The evolution of life histories. J Evol Biol.

Schluter D. 1996. Adaptive radiation along genetic lines of least resistance. Evolution. 50(5):1766–1774.

Seehausen O, Wagner CE. 2014. Speciation in Freshwater Fishes. Annu Rev Ecol Evol Syst. 45(1):621–651.

Shama LNS. 2015. Bet hedging in a warming ocean: predictability of maternal environment shapes offspring size variation in marine sticklebacks. Glob Chang Biol. 21(12):4387–4400.

Shama LNS, Mark FC, Strobel A, Lokmer A, John U, Wegner KM. 2016. Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. Evol Appl. 9(9):1096–1111.

Shama LNS, Strobel A, Mark FC, Wegner KM. 2014. Transgenerational plasticity in marine sticklebacks: maternal effects mediate impacts of a warming ocean. Funct Ecol. 28(6):1482–1493.

Sibly RM, Atkinson D. 1994. How Rearing Temperature Affects Optimal Adult Size in Ectotherms. Funct Ecol. 8(4):486–493.

Skúlason S, Parsons KJ, Svanbäck R, Räsänen K, Ferguson MM, Adams CE, Amundsen P-A, Bartels P, Bean CW, Boughman JW, et al. 2019. A way forward with eco evo devo: an extended theory of resource polymorphism with postglacial fishes as model systems. Biol Rev Camb Philos Soc. 94(5):1786–1808.

Snorradóttir RG. 2023. Relationships between phenotype, diet, and individual specialization of threespine stickleback. Kristjánsson BK, editor. MSc thesis, Hólar University.

Stearns SC. 1998. The evolution of life histories. Oxford University Press.

Strickland K, Räsänen K, Kristjánsson BK, Phillips JS, Einarsson Á, Snorradóttir RG, Bartrons M, Jónsson ZO. 2023. Genome-phenotype-environment associations identify signatures of selection in a panmictic population of threespine stickleback. Mol Ecol. 32(7):1708–1725.

Sultan SE, Spencer HG. 2002. Metapopulation structure favors plasticity over local adaptation. Am Nat. 160(2):271–283.

Swarup H. 1958. Stages in the development of the stickleback *Gasterosteus aculeatus* (L.). Development. 6(3):373–383.

Taugbøl A, Junge C, Quinn TP, Herland A, Vøllestad LA. 2014. Genetic and morphometric divergence in threespine stickleback in the Chignik catchment, Alaska. Ecol Evol. 4(2):144–156.

West-Eberhard MJ. 1989. Phenotypic Plasticity and the Origins of Diversity. Annu Rev Ecol Syst. 20(1):249–278.

Wickham H. 2016. Data Analysis. In: Use R! Springer International Publishing. p. 189–201.

Wootton RJ. 1973. Fecundity of the three-spined stickleback, *Gasterosteus aculeatus* (L.). J Fish Biol. 5(6):683–688.

Wootton RJ. 1977. Effect of Food Limitation During the Breeding Season on the Size, Body Components and Egg Production of Female Sticklebacks (*Gasterosteus aculeatus*). J Anim Ecol. 46(3):823–834.

Zuo W, Moses ME, West GB, Hou C, Brown JH. 2012. A general model for effects of temperature on ectotherm ontogenetic growth and development. Proc Biol Sci. 279(1734):1840–1846.

4 Changes in allometry and gene expression in threespine stickleback (*Gasterosteus aculeatus*) in response to temperature and diet

Alessandra Schnider [1,2], Bjarni K. Kristjánsson [1], Katja Räsänen [3], Kasha Strickland [4], Zophonías O Jónsson[2]

[1] Department of Aquaculture and Fish Biology, Hólar University, Iceland
[2] Faculty of Life and Environmental Sciences, University of Iceland, Iceland
[3] Department of Biological and Environmental Science, University of Jyväskylä, Finland
[4] Institute of Ecology and Evolution, School of Biological Sciences, University of Edinburgh, UK

4.1 Abstract

Rapid environmental changes can be challenging, but most organisms can to some degree mitigate adverse effects through phenotypic plasticity, the ability of a single genotype to produce multiple phenotypes in response to environmental stimuli. The most common mechanism through which plasticity is achieved is differential gene expression. Threespine stickleback (*Gasterosteus aculeatus*) have served as a model organism to study environmentally induced phenotypic variation and rapid changes in phenotypes within and across generations. The aim of this project was to study variation in allometry and gene expression in the spatio-temporally varying system, Lake Mývatn threespine stickleback. To this end we reared first-generation offspring of wild caught stickleback under differing experimental conditions (temperature: 12°C vs. 22°C; diet: epibenthic vs. benthic). At sexual maturation we collected body size measurements (standard length, head length and weight) and liver samples. We also collected liver samples from wild breeding males and females from contrasting habitats (Cold Shore vs. Hot Shore). We found differences in allometry in response to the contrasting temperature and diet treatments. Notably, relative head size was bigger in individuals in warm temperatures, and on an epibenthic diet. We also found strong signals of differential gene expression in response to the contrasting rearing conditions. In response to temperature, we identified various differentially expressed genes associated with heat stress and immune response in experimental and wild individuals alike, indicating that warm temperatures are challenging. Experimental females additionally showed differential gene expression in response to diet. An epibenthic diet appeared to have an antioxidative effect, potentially mitigating adverse effects of warm temperatures. In wild individuals this could be especially beneficial when dispersing into the warm habitat within Lake Mývatn. Thus, this study deepens our understanding of the relevance phenotypic plasticity in a the highly dynamic system of Lake Mývatn threespine stickleback and gives us insight into the molecular mechanisms involved.

4.2 Introduction

Genetic divergence in response to environmental selection pressures is a major contributor to phenotypic diversity (Skúlason et al. 2019). However, trading fixed genetic responses for the ability to plastically adapt to environmental cues is costly and thus only favoured when environmental changes are rapid over space and/or time, and if variation is predictable (Sultan and Spencer 2002; Fawcett and Frankenhuis 2015) and costs of phenotypic plasticity are low (Pfennig 2021). Phenotypic plasticity - the ability of a genotype to express different phenotypes in response to environmental stimuli - can be mediated through gene expression (West-Eberhard 2003; Pfennig 2021).

Indeed, we are just beginning to understand the complex molecular interactions of genes and their role in phenotypic variation, which is further complicated as many genes have organ specific functions and tissue specific transcripts and isoforms (Singaraja et al. 2005). Moreover, phenotypic plasticity in response to current environmental conditions, parental, or even grandparental environmental conditions can affect offspring phenotype (Shama et al. 2014; Pfennig 2021). Transgenerational effects have been reported in many species, in response to a wide range of environmental conditions and phenotypes. Thus, studies integrating multiple environmental factors across multiple generations can be useful to understand observed patterns in the wild.

In ectotherms some of the major drivers of phenotypic plasticity are temperature and diet. An increase in temperature can change body allometry with relative head size getting smaller with increasing temperatures. This has been seen in various fish species such as threespine stickleback (*Gasterosteus aculeatus*), Eurasian perch (*Perca fluviatilis L.*), goldfish (*Carassius auratus*), white perch (Morone americana) and green sunfish (*Lepomis cyanellus*) (Olsson et al. 2007; Chivers et al. 2008; Chizinski et al. 2010; Ramler et al. 2014). Fish have also been shown to invest relatively more into head growth when energetically limited, as can be the case under increased temperatures (Ramler et al. 2014) or due to dietary limitations (Olsson et al. 2007; Chivers et al. 2008; Chizinski et al. 2010). Furthermore, metabolic rates increase with temperature and consequently, studies investigating energetic limitations have focused on tissues directly related to metabolic rate, such as the liver (Castro et al. 2012; Killen et al. 2016; He et al. 2023).

Environmental effects can also span generations, for example via epigenetic inheritance such as alternative methylation or maternal effects, which are often mediated through the egg yolk. Egg yolk is a critical source of nutrients and energy for the developing embryo, and the amount of yolk deposited in the egg is directly correlated with size at hatching (Leblanc et al. 2016; Leblanc et al. 2023). The main precursor protein of egg yolk is Vitellogenin which is produced in the liver of females and transported to the ovaries through the bloodstream (Yilmaz et al. 2024). The amount of Vitellogenin a female produces is therefore a direct measurement of her maternal investment. Consequently, an individual's phenotype is not necessarily just the product of its own environment but might also reflect its parents' environment.

Isolated and relatively simple ecosystems offer a unique opportunity to understand how habitat variation can shape phenotypic variation, within and across generations, and elucidate the importance of differential gene expression in the process. One such opportunity can be found in threespine stickleback inhabiting the highly dynamic system of Lake Mývatn, Iceland. From previous studies we know that threespine stickleback in this population show considerable phenotypic variation in response to temperature (e.g. body size) and diet (e.g. gill raker number and length) (Millet et al. 2013; Strickland et al. 2023). Some of this variation can be attributed to genetic variation (Strickland et al. 2023) and multiple traits appear to respond to selection (Strickland et al. 2024), but there is still phenotypic variation that cannot be accounted for by

genetic variation. Therefore, differential gene expression could be central to the observed phenotypic variation in this population.

The goal of this study was to investigate the impact of temperature and diet on differential gene expression within and across generations. We further tested for potential energetic challenges associated with varying temperatures and diets by examining overall body size, condition, and allometry, which may provide insight into resource allocation during development. To investigate this, we conducted a plasticity experiment where we crossed individuals from two contrasting habitats within Lake Mývatn and incubated and reared their offspring in contrasting temperatures and diet treatments until sexual maturity (Fig. 4.1).

Based on previous results (**Paper II**) we did not expect any effects of parental origin within Lake Mývatn in first generation individuals. Instead, we expected temperature to be the primary driver of differential gene expression, with pathways related to metabolism, immune response, and heat stress to be upregulated in warm experimental temperatures compared to cold temperatures. In response to contrasting diets, we expected pathways related to metabolism to be affected by differential gene expression to deal with the varying nutrient compositions of diets. Furthermore, we expected relative head size to reflect resource limitation as indicated in **Paper II** by the lower body condition in females reared in warm rearing temperatures and on an epibenthic diet. Moreover, in **Paper II** we found clear differences in maternal investment in females based on rearing temperatures, thus, we expected gene expression of vitellogenin to reflect this.

4.3 Methods

4.3.1 Study system

Lake Mývatn (65°36'N, 17°00'W; 278m a.s.l.) in north-eastern Iceland is a shallow eutrophic lake, that varies greatly in a range of abiotic and biotic factors (**Paper II** Fig. 3.1) (Einarsson et al. 2004). Its 37km^2 area is divided into two main basins connected by two narrow channels: the smaller, spatially more heterogeneous and deeper (1-6m depth) north basin and the more homogenous and shallower (2-3.2m depth) south basin (Einarsson and Örnólfsdóttir 2004; Phillips et al. 2023). The lake greatly varies in productivity, invertebrate abundance and community structure in both space and time (Einarsson and Örnólfsdóttir 2004; Ives et al. 2008; Bartrons et al. 2015). Based on temperature, depth, substrate and vegetation, five habitats inhabited by stickleback had previously been defined as warm, mined, rocky shore, Cladophorales and pondweed (Millet et al. 2013).

Here we focused on two environmentally contrasting habitats, "Warm" and "Rocky Shore" (from here on referred to as "Hot Shore" and "Cold Shore"). Hot Shore is dominated by lava rock and silica substrate with little vegetation (pondweed *Potamogeton filiformis*) and warm springs, which keeps this part of the lake from freezing during winter. The annual temperature variation is relatively low with mean temperatures in summer 19.9 ± 1.8°C and 16.2 ± 2.2°C in winter. Threespine stickleback in this habitat have a more mixed diet with a large proportion of Chironomidae larvae (Millet 2013). Cold Shore shows much greater seasonal fluctuations with mean temperatures in summer 11.6 ± 1.8°C and 3.3 ± 2.7°C in winter. The temperature in Cold Shore is largely dependent on ambient temperature and therefore, it freezes over completely during winter. Furthermore, Cold Shore is characterized by a rocky substrate and vegetation of watermilfoil (*Myriophyllum spicatum*) (Millet et al. 2013). Stickleback in this habitat predominantly feed on various Cladocera species (Millet 2013).

4.3.2 Collection of breeding fish

This study involved the lethal sampling of wild animals, bringing live animals to rearing facilities and subjecting them to experimental condition. All experimental work was conducted in accordance with Icelandic laws and regulations set forth by the Icelandic animal care authorities (animal care permit number: 2020-03-02). Lake Mývatn is a protected area, thus, all sampling was done in collaboration with RAMÝ (The Mývatn Nature Research Station), and the local landowners. In June 2020 threespine stickleback in breeding condition were collected from the two habitats in Lake Mývatn, Hot Shore and Cold Shore, using unbaited minnow traps. The traps stayed out overnight for approximately 12 hours at each site. The fish were then transported in oxygenated tanks from Mývatn to the research facilities at Verið, the main research facility of the Department of Aquaculture and Fish Biology of Hólar University in Sauðárkrókur. There they were kept in 27L sex-origin specific holding tanks at approximately 18°C and fed frozen midge larvae twice a day under 24 hours light, to maintain and stimulate breeding conditions, until crosses were made.

We additionally collected wild adult breeding fish from Hot Shore and Cold Shore in July 2021 to be included in the gene expression study. We collected four males and four females from each location and transferred them to the research facilities of Hólar University. All adults were kept in 27L holding tanks (one for each sex and origin respectively) at approximately 18°C and under constant light. All individuals were sampled within 24h of their arrival at the research station and had received no food during their holding period.

4.3.3 Experimental design

We conducted a rearing experiment with threespine stickleback from two contrasting locations within Lake Mývatn serving as the source population. After artificially crossing wild individuals, their offspring were reared at contrasting temperatures (12°C vs. 22°C) and diets (benthic vs. epibenthic) until sexual maturity at which point all adults were terminally sampled (Fig. 4.1).

4.3.4 Crossing and rearing conditions

A total of 34 full sibling families (Cold Shore N = 18, Hot Shore N = 16) were created by artificially crossing wild-caught individuals over the course of two weeks (see **Paper II** for details). Approximately one hour post fertilization the F1 clutches were split in half, placed in individual breeding cups (plastic cups with mesh bottoms) floating in a 15L tank in a flow-through system and incubated at two contrasting temperatures (12°C vs. 22°C). Eggs were checked daily, and dead eggs were removed. Once a sufficient number of successful crosses were obtained, all remaining adults were terminally sampled and processed as part of the adult sampling protocol (see **Paper II**).

Shortly after the majority of individuals in a clutch reached swim-up stage (yolk sac is absorbed and fish start actively swimming and foraging), all individuals were counted and released into the rearing tank. All individuals were fed live *Artemia salina* nauplii ad libitum twice a day, seven days a week. To account for differences in growth rate, fish were kept for two weeks in the warm treatment and four weeks in the cold before being split into two evenly sized groups. Each group was then transitioned to either a benthic or epibenthic diet treatment and fed ad libitum. Each 15L tank contained on average 22 (9-35) individuals and all tanks were enriched with an artificial plant and two petri dishes filled with sand.

Fish from the epibenthic treatment were fed with a mix of Cladocera (*Chydorus sphaericus* and *Daphnia sp.*, cultured in our research facilities) at the water surface, whereas fish from the benthic treatment were fed commercially available frozen midge (Chironomidae) larvae in a sand dish at the bottom of the tank. Initially midge larvae were grated or chopped until fish were big enough to ingest entire midge larvae. However, we were not able to produce enough Cladocera to sustain all the fish on a pure epibenthic diet, therefore we supplemented with *Artemia salina* nauplii. Additionally, all fish received commercial pellets (INICIO Plus G by BioMar); initial pellet size was 0.4mm, but with increasing body size fish were transitioned to a bigger pellet size of 0.5mm). The pellets were composed of crude protein (62%), crude fats

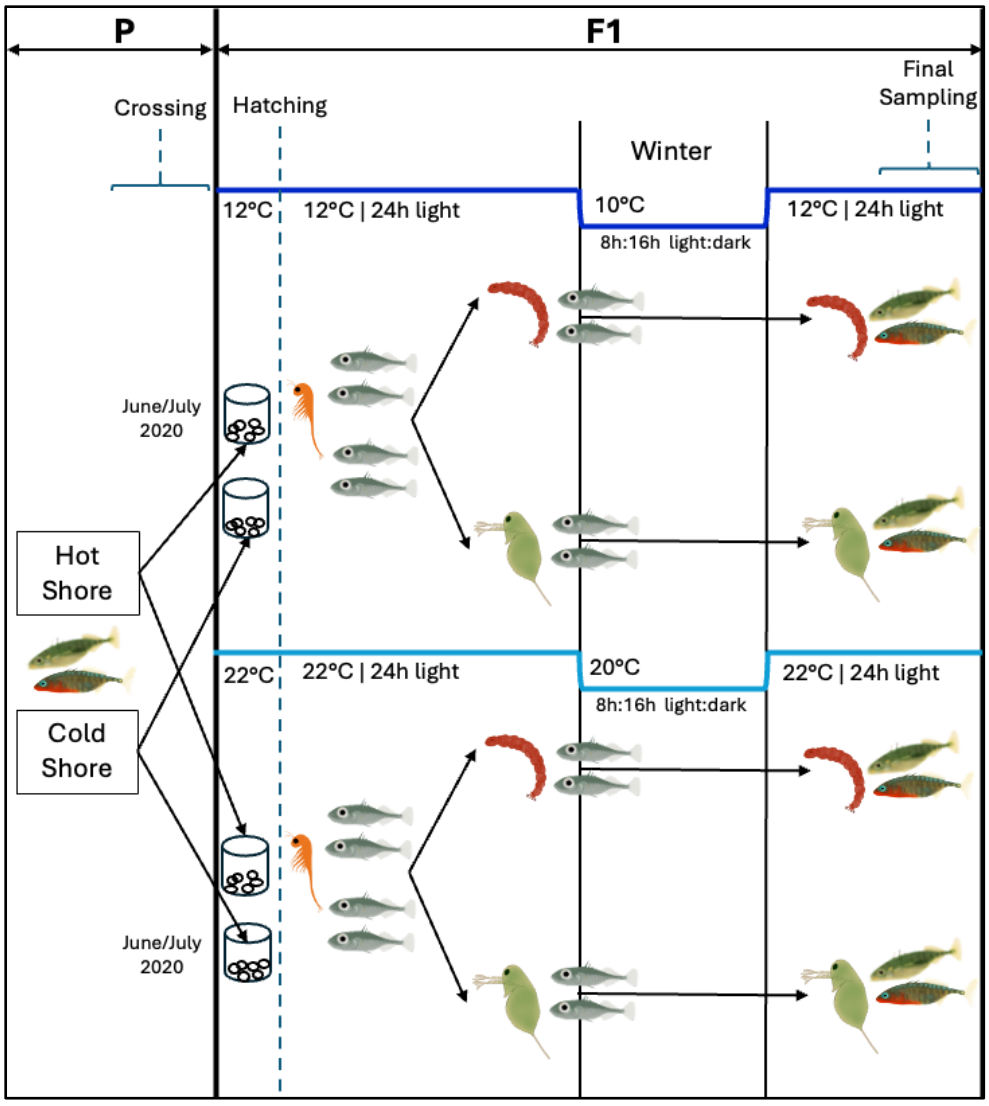

Figure 4.1 *Illustration of the design of a plasticity experiment conducted on threespine stickleback (Gasterosteus aculeatus) from Lake Mývatn Iceland, originating from two locations "Hot Shore" and "Cold Shore" within the lake. It includes illustrations of fish at juvenile and adult sampling stage as well as food items used in this experiment (orange: Artemia salina; red: Midge (Chironomidae) larvae; green: Cladocera spp.). Artemia salina were used post hatching until the experimental fish were split into their respective diet treatments; benthic diet: Midge larvae and epibenthic diet: Cladocera spp. supplemented with Artemia salina. It also depicts the light and temperature regime across the whole experimental period as well as sampling events.*

(10%), crude fibre (0.2%), Ash (10.3%), Phosphorus (1.53%), Calcium (2.16%), Sodium (0.75%) which ensured, that all fish received all the essential nutrients, irrespective of their diet treatment. Until juvenile sampling, fish received 12 live feeds over six days a week and pellets once a week.

At 1300 degree-days (DD; 60 days in warm and 110 days in cold rearing temperatures) tank density was reduced to 20 individuals per tank, to avoid overcrowding. We chose degree days as measurement to standardize between developmental stages across temperatures, as development is greatly accelerated in warm temperatures (Sibly and Atkinson 1994; Angilletta et al. 2004; Kuparinen et al. 2011). Furthermore, we chose this time point because previous studies have shown that by 1200 DD the stickleback has fully developed its skeletal structure and thereafter only grows in size (Currey et al. 2017). Two weeks after adjusting tank densities all fish were transitioned to winter conditions for four months in both temperature treatments. During winter conditions temperatures were reduced by 2°C (10°C vs. 20°C) and a photoperiod (8h:16h light:dark) introduced. Further, the number of live feeds was reduced to seven per week (spread over 4 days), pellets were fed twice a week, and a break day was introduced, where fish did not receive any food.

After the four-month period, the fish were transitioned to a 24h light period, to stimulate and synchronize sexual maturation. Feeding was changed to five live feeds over three days, three pellet feeds whilst still including a rest day. Further, an additional artificial plant was added to each tank to reduce aggression among males due to the breeding season and the sand was removed to discourage males from building nests. Once most of the tanks contained reproductively active females and males displaying breeding colouration, adult sampling commenced.

4.3.5 Sample collection and body size and allometry measurements and analyses

A total of 459 sexually mature individuals across all treatment conditions were terminally sampled in two major sampling events separately by rearing temperatures. In March/April 2021 over a time span of 53 days, 118 females and 114 males from the warm temperature treatment were sampled. Subsequently in May/June 2021, 116 females and 111 males from the cold rearing treatment were sampled within 18 days (Table 4.1). On the day of sampling, all fish were starved and terminally sampled following our standard protocol (see **Paper II**).

Body size and allometry measurements were based on images of the left body side that were converted into *tps* files using *tpsUtil*. To measure body length, we used two fixed landmarks (subset of a total of 33 landmarks as used in Snorradóttir (2023)) in *tpsDig2* v.2.32, namely at the tip of the lower lip and in the middle of the posterior end of vertebrae (McGee et al. 2013; Taugbøl et al. 2014). Head length was measured from the tip of the lower lip to the posterior extent of the supraoccipital bone dorsal section between the frontal and the supraoccipital bone, identified as a small depression in the head.

Table 4.1 *Sample sizes of first generation adult threespine stickleback (Gasterosteus aculeatus) reared in a plasticity experiment under contrasting temperatures (cold:12°C vs. warm 22°C) and diets (epibenthic vs. benthic). Origin refers to the parental origin within Lake Mývatn, Iceland (Cold Shore vs. Hot Shore). Body size indicates sample sizes of sexually mature F1 adults for which body length, head length and body condition were measured. RNA indicates number of livers collected from reproductively active F1 adults for RNA sequencing.*

Temperature	***Diet***	***Origin***	***Females***		***Males***	
			Body Size	***RNA***	***Body Size***	***RNA***
Cold	**Benthic**	**Cold shore**	34	3	30	3
		Hot shore	25	5	24	5
	Epibenthic	**Cold shore**	31	3	30	3
		Hot shore	26	5	27	5
Warm	**Benthic**	**Cold shore**	30	3	30	5
		Hot shore	29	5	24	5
	Epibenthic	**Cold shore**	30	5	30	5
		Hot shore	29	5	30	5

We performed a generalized Procrustes analysis using the "gpagen" function from the *geomorph* package (Baken et al. 2021; Adams et al. 2024) in *R* (Posit team 2023) to rotate, scale and align landmarks. This produced eight shape variables (X and Y coordinates for four landmarks) which were used in the "interlmkdist" function from the *geomorph* package to measure inter-landmark distance, producing standard body and head length measurements. As head length is strongly correlated with body size, we used the residuals of a simple linear model with head length as the response variable and body size as the fixed effect. The linear model was run using the "lm" function and residuals extracted with the "resid" function both part of the *stats R package* (R Core Team 2023).

Finally, body condition was calculated with weight, post stripping in case of gravid females, and standard body length using Fulton's condition factor (Nash et al. 2006). Applying linear mixed-effects models using the *lmer* function of the *lme4* package (Bates et al. 2014) we tested for differences in body length, residual head length and body condition among the treatment combinations. Thus, all models contained temperature, diet, origin and their one-way interactions, and sex as fixed effects. We further included tank identity nested within family identity as random effects in all models to correct for family and tank wise effects. We also tested for an effect of sampling date in the F1 adults, due to the extended sampling period in the warm temperature treatment (warm: 53 days vs. cold: 18 days). However, we did not find any significant effect of sampling date on standard length (SL) ($F = 0.11$, $p = 0.743$, Supplementary Fig. B.1) and thus, we did not include it in the final model. Consequently, the final model to assess standard body length, body condition and residual head length was: ~ Temperature * F1 Diet + Temperature * Origin + F1 Diet * Origin + Sex + (1|Family/Tank ID). β and standard errors were extracted using the *base R* "summary" function (R Core Team 2023). Residual degrees of freedom, F and p-values were obtained with the "Anova" function (statistic = F and type = II) from the *car* package (Fox and Weisberg 2018).

4.3.6 RNA sample collection and sequencing

We decided to collect RNA samples from livers, as this organ is central to various metabolic processes such as processing of glucose and lipids, as well as the storage of lipids in many fish species. Moreover, previous studies found differential gene expression in liver tissues in response to temperature (Oomen and Hutchings 2017). Large increases in temperature can result in severe stress responses, such as the accumulation of oxidative damage products in the liver (Carney Almroth et al. 2015). Additionally, diet has been shown to be able to mitigate some of the harmful effects of elevated temperature, such as oxidative stress (Castro et al. 2012).

All RNA samples were collected as part of the standardized crossing protocol to assure that all individuals were in comparable reproductive conditions (see **Paper II**). Once fish were euthanized and sperm and eggs collected, livers were dissected out and submerged in DNA/RNA Shield (Zymo Research) in a 1.5ml Eppendorf tube. The tubes were left at room temperature for 24h and then stored at -20°C until further processing. We sampled livers from a total of 16 wild caught (4 per origin x sex) and 70 lab reared individuals (Table 4.1). Our target was five replicates per sex x treatment x origin group to ensure balanced representation and control for family effects. However, we struggled to obtain samples from females originating from "Hot Shore" reared in cold temperatures, due to lower number of families in this treatment group.

In July 2021 all RNA samples were sent to BGI Tech Solutions (BGI 2020) for RNA extraction and RNA sequencing. Approximately 50M clean paired end 100 bp reads were obtained per sample. RNAseq reads were pseudo-aligned to the *Gasterosteus aculeatus* genome assembly version 5 (Nath et al. 2020) using Kallisto (Bray et al. 2016) to obtain transcript abundance per sample on an isoform-level using gene annotations from the ENSEMBL "*gaculeatus_gene_ensembl*" data set (Harrison et al. 2024).

4.3.7 Differential gene expression and gene ontology analyses

We used the R package *sleuth* to identify differentially expressed genes as it was developed by the same group that developed Kallisto (Pimentel et al. 2017; R Core Team 2023; Pimentel and Mcgee 2024). Sleuth accounts for variability between replicates due to the pseudoalignment approach Kallisto employs and is therefore a robust option to assess differential gene expression. We directly imported the read count per transcript obtained from Kallisto to test for the effects of temperature, diet, and origin within Lake Mývatn on gene expression. The threespine stickleback reference genome on ENSEMBL (https://oct2024.archive.ensembl.org, ENSEMBL Release 114, October 2024) was obtained with the R package *biomaRt* (Durinck et al. 2005; Durinck et al. 2009). We found considerable differences in the gene expression patterns between sexes, thus we decided to further test the effects of temperature, diet, and origin in each sex separately as we were primarily interested in the effects of environmental conditions rather than sex differences in gene expression.

We fitted a full linear model containing all fixed effects (e.g. temperature, diet, origin) with the *sleuth_fit* function from the R package ***sleuth***. Consequently, for lab reared individuals the model included rearing temperature, diet, and origin. For wild caught individuals the model consisted of origin only. We then used a Wald test by applying the function *sleuth_wt* to the sleuth model to test for the effects of one of the environmental variables at the time and to obtain fold change values. Finally, we filtered for reads with an adjusted p-value < 0.05. We extracted unique ensembl ids to have a count of differentially expressed genes, which we subsequently used for gene ontology (GO) analyses.

We used the *R* package *topGo* (Alexa and Rahnenfuhrer 2024) to conduct the functional enrichment analysis. We extracted the threespine stickleback specific GO terms from the ENSEMBL data base and specified the gene ontology as "*Biological Processes*" (BP). To reduce false positives, we pruned the GO hierarchy by requiring that each GO term had at least 10 annotated genes in our reference list ("nodeSize = 10"). Finally, we identified genes of interest through multiple approaches. We looked for outliers in volcano plots, that clearly stood out from the general distribution either due to a high log fold change or an exceptionally low adjusted p-value. We further looked at genes that were differentially expressed in both wild and experimental fish.

4.4 Results

4.4.1 Body size and allometry

Standard body length was significantly impacted by temperature (cold:12°C vs. warm:22°C) and diet (epibenthic vs. benthic) as well as an interaction between the two variables (Table 4.2). Fish reared in the warm temperature treatment and on a benthic diet were larger than individuals reared in the cold temperature treatment and on an epibenthic

diet. Furthermore, the difference in size between diet treatments was more pronounced in the warm temperature treatment than in the cold, indicated by the interaction term. There was, however, no difference between sexes (Table 4.2). Relative head size was different between temperature and diet treatment as well as between the sexes (Table 4.2). Fish reared in the warm treatment, and fish reared on an epibenthic diet had bigger heads relative to their standard body length (Fig. 4.2A). Furthermore, males had relatively bigger heads than females (Fig. 4.2A).

Body condition differed depending on diet treatment, whereas temperature had no effect (Table 4.2). Body condition was higher on a benthic diet in both sexes (Fig. 4.2B). However, we detected a temperature-diet interaction (Table 4.2). The difference in body condition was more pronounced in the cold temperature treatment in both sexes (Fig. 4.2B). Furthermore, sex had the biggest effect on body condition (Table 4.2). Males had a higher body condition than females irrespective of treatment condition (Fig. 4.2B). Origin within Lake Mývatn (Cold Shore vs. Hot Shore) had no effect on body size, condition factor or in head size in the here presented experiment (Table 4.2).

4.4.2 Differential gene expression and top GO terms

The majority of the differentially expressed genes (DEGs) in both experimental sexes were in response to rearing temperature, however only about half of these were shared between the sexes (Fig. 4.3). In addition, in females we found a considerable number of DEGs in response to diet, approximately half of which also showed a temperature response (Fig. 4.3A). In males, diet did not elicit a strong response in gene expression. Only a total of two genes were differentially expressed in males in response to diet and of those two only one was uniquely attributed to diet effects. The other gene overlapped with temperature (Fig. 4.3B). Furthermore, an effect of parental origin within Lake Mývatn was found for 17 DEGs in males, of which six overlapped with temperature, leaving 11 DEGs specific to parental origin (Fig. 4.3B)

In the wild caught adults, we identified almost six times the number of DEGs in males than in females (Supplementary Fig. C.1). In wild caught males, 518 genes were differentially expressed between habitats of which 300 were also differentially expressed in laboratory males in response to experimental temperature. None of the DEGs in wild males overlapped with DEGs in response to diet or origin. In the wild caught females, we only detected 90 genes differentially expressed between habitats, of which 57 were also differentially expressed in laboratory females in response to experimental temperature and nine in response to diet. Nine genes that were differentially expressed between origins in wild females were also differentially expressed in wild males.

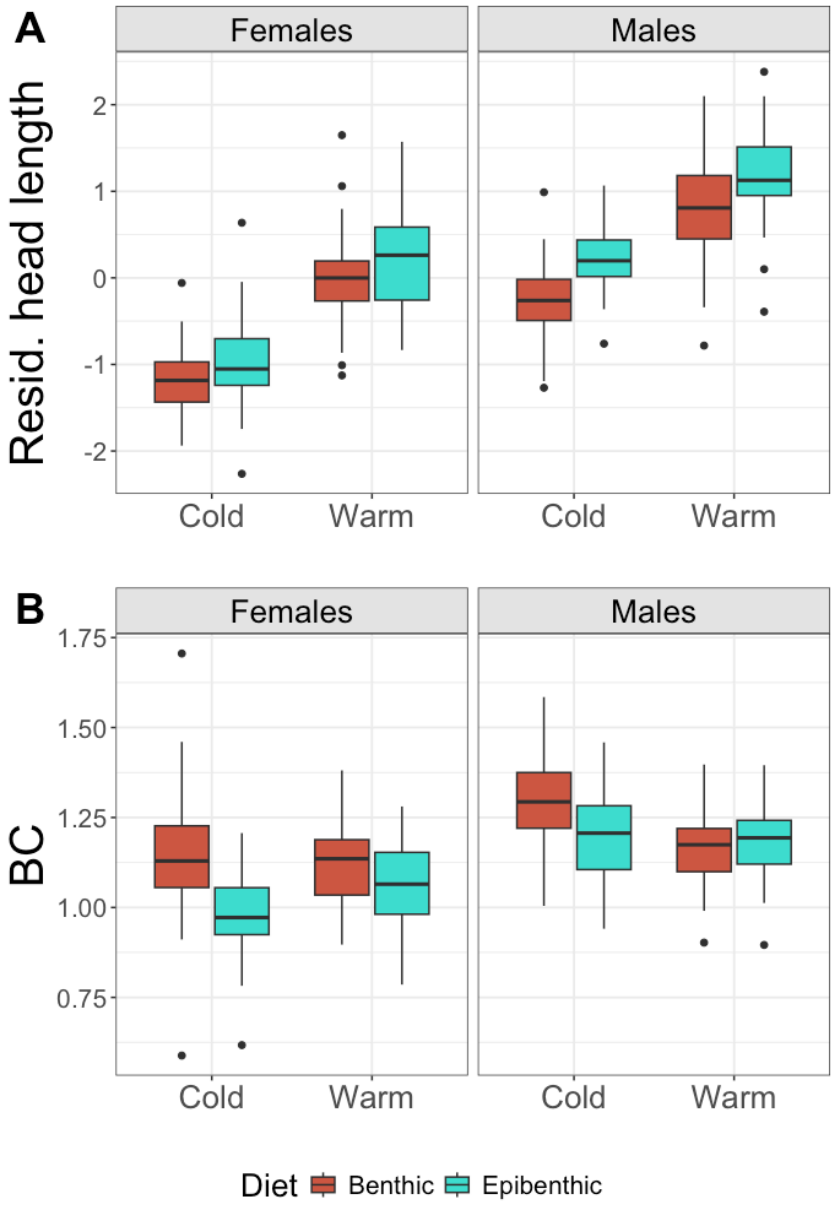

Figure 4.2 *F1 sexually mature female and male threespine stickleback (Gasterosteus aculeatus) reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (epibenthic vs.benthic) (see Fig. 4.1). Individual points represent measurements of individual females and males. A) Relative head size (mm) of females and males was described by using the residuals (Resid.) from a simple linear model (lm function from the stats package in R Studio* (R Core Team 2023)*) with head length as a response variable and standard length (SL) as a fixed effect. B) Body condition (BC) (Fulton's K;* (Nash et al. 2006) *of females and males was calculated using SL and body weight. In case of gravid females, we used the weight after the female had released the eggs. Boxplots show the median (horizontal line), interquartile range (box), and 1.5× interquartile range whiskers; each dot represents an individual measurement outside this range (outliers).*

Table 4.2 *Results of linear mixed-effects models assessing temperature and diet effects on first-generation Threespine stickleback from a plasticity experiment with contrasting temperatures (12°C vs. 22°C) and diets (epibenthic vs. benthic) (Fig. 3.2). Origin refers to parental origin within Lake Mývatn (Cold Shore vs. Hot Shore) (Fig. 3.1). Residual standard deviation, β, and standard errors were extracted using base R's „*summary*" function (R Core Team 2023), while residual degrees of freedom, F, and p-values were obtained via the* Anova *function (type II, statistic = F) from the* car *package (Fox & Weisberg, 2018).*

Focal trait	*N*	*Model type*	*Random effects*	*Fixed effects*	*b ± SE*	*DF res.*	*F*	*Type II p-value*
Standard length	459	lmer	Family ID/Tank ID	**Temp.**	**3.01 ± 0.53**	10.3	**32.45**	**< 0.001**
			Residual SD = 2.79	**Diet**	**-1.59 ± 0.45**	443.2	**68.52**	**< 0.001**
				Origin	0.17 ± 0.83	20.5	1.66	0.212
				Sex	-0.11 ± 0.26	433.1	0.18	0.670
				Temp. * Diet	**-1.91 ± 0.54**	448.6	**12.30**	**< 0.001**
				Temp * Origin	0.48 ± 0.76	8.83	0.35	0.568
				Diet * Origin	0.59 ± 0.54	444.3	1.17	0.280
Res. head length	459	lmer	Family ID/Tank ID	**Temp.**	**1.10 ± 0.08**	**10.3**	340.66	**< 0.001**
			Residual SD = 0.44	**Diet**	**0.39 ± 0.07**	**443.6**	64.80	**< 0.001**
				Origin	-0.08 ± 0.13	20.4	0.86	0.365
				Sex	**0.99 ± 0.04**	**433.5**	572.76	**< 0.001**
				Temp. * Diet	-0.03 ± 0.09	449.0	0.13	0.720
				Temp * Origin	0.04 ± 0.12	8.8	0.12	0.734
				Diet * Origin	-0.06 ± 0.08	444.7	0.56	0.454
Body Condition	459	lmer	Family ID/Tank ID	Temp.	-0.07 ± 0.02	11.6	0.08	0.781
			Residual SD = 0.11	**Diet**	**-0.12 ± 0.02**	442.2	**60.26**	**< 0.001**
				Origin	0.02 ± 0.04	20.8	0.19	0.666
				Sex	**0.13 ± 0.01**	430.2	**173.8**	**< 0.001**
				Temp. * Diet	**0.11 ± 0.02**	445.4	**27.3**	**< 0.001**
				Temp * Origin	0.01 ± 0.04	11.3	0.01	0.800
				Diet * Origin	-0.02 ± 0.02	443.0	1.1	0.300

Hierarchical clustering based on DEGs with an adjusted $p < 0.0001$ in response to temperature successfully distinguished between cold and warm rearing conditions in the experimental individuals. The wild caught females from both origins clustered with warm reared experimental individuals, whereas the Hot Shore males clustered with warm reared males and males from Cold Shore clustered with cold experimental males (Fig. 4.4). Moreover, experimental individuals exhibited more extreme expression patterns while wild individuals displayed more intermediate expression patterns (Fig. 4.4). The pattern in the heatmap based on experimental diet and origin habitats was more complex. The hierarchy showed two clusters for each respective experimental diet treatment group (Supplementary Fig. C.2). Furthermore, wild caught individuals clustered with both diet treatments, apparently irrespective of origin within Lake Mývatn.

The top 12 GO terms in response to experimental rearing temperature all contained at least 30% of genes within the gene set and were therefore well supported (Fig. 4.5A & 4.5B). They primarily encompassed biological processes related to various aspects of development, growth, and cellular functions in both males and females. For females one of the top GO terms was "Embryonic morphogenesis" which is fitting since we collected the liver samples at spawning and the liver produces vitellogenin, the major precursor protein which forms the egg yolk. Indeed, one of the genes involved in the Vitellogenin production (*vtg3*) was differentially expressed between experimental females reared in contrasting temperatures (Fig. 4.6A). In females the top GO terms in response to experimental diet contained 10% – 40% of the genes in the gene set (Fig. 4.5C). They are mostly in connection to responses to stimuli and developmental processes in various organs and morphology. Since in males two genes were differentially expressed in response to diet, a GO analysis was not feasible (Fig. 4.3B).

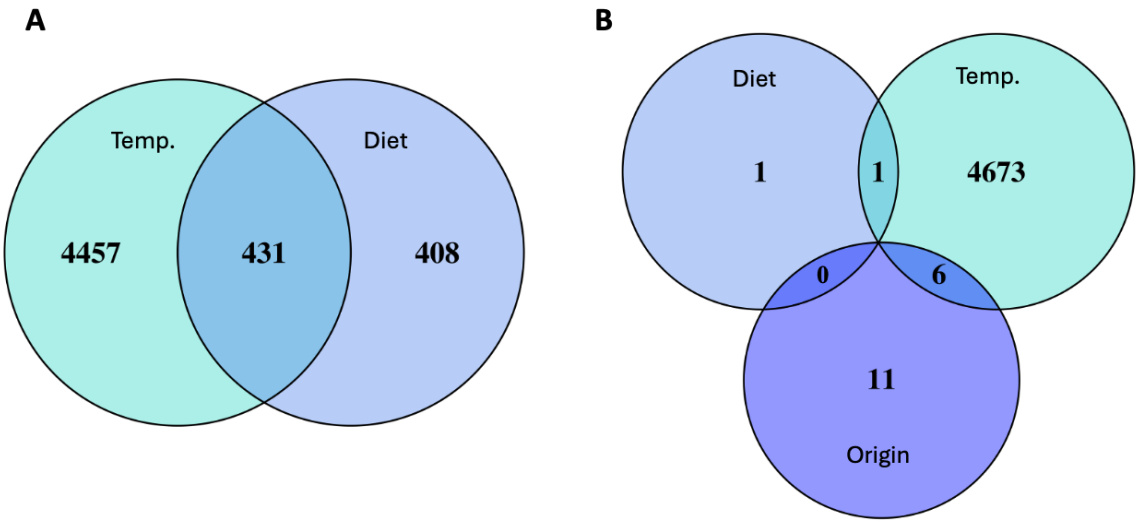

Figure 4.3 *Number of differentially expressed genes (DEGs) in livers from first generation threespine stickleback (Gasterosteus aculeatus) reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (epibenthic vs. benthic) (see Fig. 4.1). Origin refers to parental origin within Lake Mývatn, Iceland in response to experimental rearing temperature, diet, and parental origin within Lake Mývatn "Hot Shore" and "Cold Shore". A) Number of DEGs in females. B) Number of DEGs in males.*

4.4.3 Genes of interest

Combining gene lists from the top 12 GO terms and genes identified in the volcano plots (Fig. 4.6), we were able to compile a list of genes of interest. For instance, in response to rearing temperature we found *DEGs* associated with translation such as ribosomal proteins (*rps2, rps27a*, *rps28*, *rps29*) and translation initiation factor (*eif* family), and genes associated with protein regulation (*psmb12, CAPN5*). Various genes associated with the Ribosomal Protein Small subunit (e.g. *rsp28*, *rsp29*) were upregulated in response to warm rearing temperatures, indicating an increase in translation. Interestingly, in both sexes the same two isoforms of the *rps27a* gene were differentially expressed and, in both sexes one isoform was upregulated, and one was downregulated (Fig. 4.6A & 4.6B). This gene encodes both for the ribosomal protein S27a and Ubiquitin (fused to the N-terminus).

Although overall genes associated with metabolic processes appear to be upregulated in response to warm temperatures, many genes specifically involved in lipid metabolism were downregulated in warm rearing temperatures (e.g. in both sexes: *apoeb, atp8a1, CAPN5*). Genes connected to the cholesterol metabolism, like *apoeb*, were of special interest, as cholesterol is a precursor for cortisol, testosterone, and estrogen, which are hormones crucial for stress response and reproduction. Finally, *vtg3* (Vitellogenin-3) was downregulated in females reared in warm experimental conditions in comparisons to those reared in the cold, thus, females in the warm produced less of the main precursor protein that makes up egg yolk. This indicates that females in warm temperatures invested less energy into their offspring compared to females in the cold (Lubzens et al. 2017).

Furthermore, we found genes involved in the response to heat stress such as the heat shock protein 90 (*hsp90aa*) and genes involved in oxidative stress connected to glutathione peroxidase (*gstt1a*, *gstr, cyp1c2*) to be upregulated in both sexes in response to warm rearing temperatures (Fig. 4.6A & 6B). The involvement of *gstt1a* in adaptation to increased water temperatures and hypoxic conditions has previously been suggested in flounder (Pédron et al. 2017). Interestingly, in stickleback females two isoforms were upregulated and one downregulated (Fig. 4.6A). We also identified upregulated genes involved in immune response (*il10ra;* interleukin, cytokines of the TNF family), apoptosis (*boka*) and ion transport (*steap4;* copper ion transport). Additionally in males we found a gene associated with detoxification (*arg2;* removes excess nitrogen from the liver) (Fig. 4.6B). (Benedicenti et al. 2017). However, we also found a downregulated genes (signal transducing adaptor: *stap2a*) in females under warm rearing conditions that was found to be involved with immune response (Fig.4.6A) (Matsuda and Oritani 2021).

When looking at specific genes in response to contrasting diet treatments in female threespine stickleback, our analyses revealed differences in gene expression related to metabolic pathways, specifically in females on an epibenthic diet we identified downregulated genes directly linked to lipid synthesis (*fads2*, *elovl6*) (Fig. 4.6C) (Xie et al. 2021). Interestingly, another gene typically involved in lipid synthesis *got1*, was upregulated in response to an epibenthic diet, but downregulated in warm rearing temperatures. However, *got1* has also been associated with ovarian development and maturation, potentially indicating an effect of temperature and diet on reproductive maturation in females (Li et al. 2023). Another gene that stood out was *aspg*, which has been found to be downregulated in response to a high fat diet (Méndez et al. 2017). In our

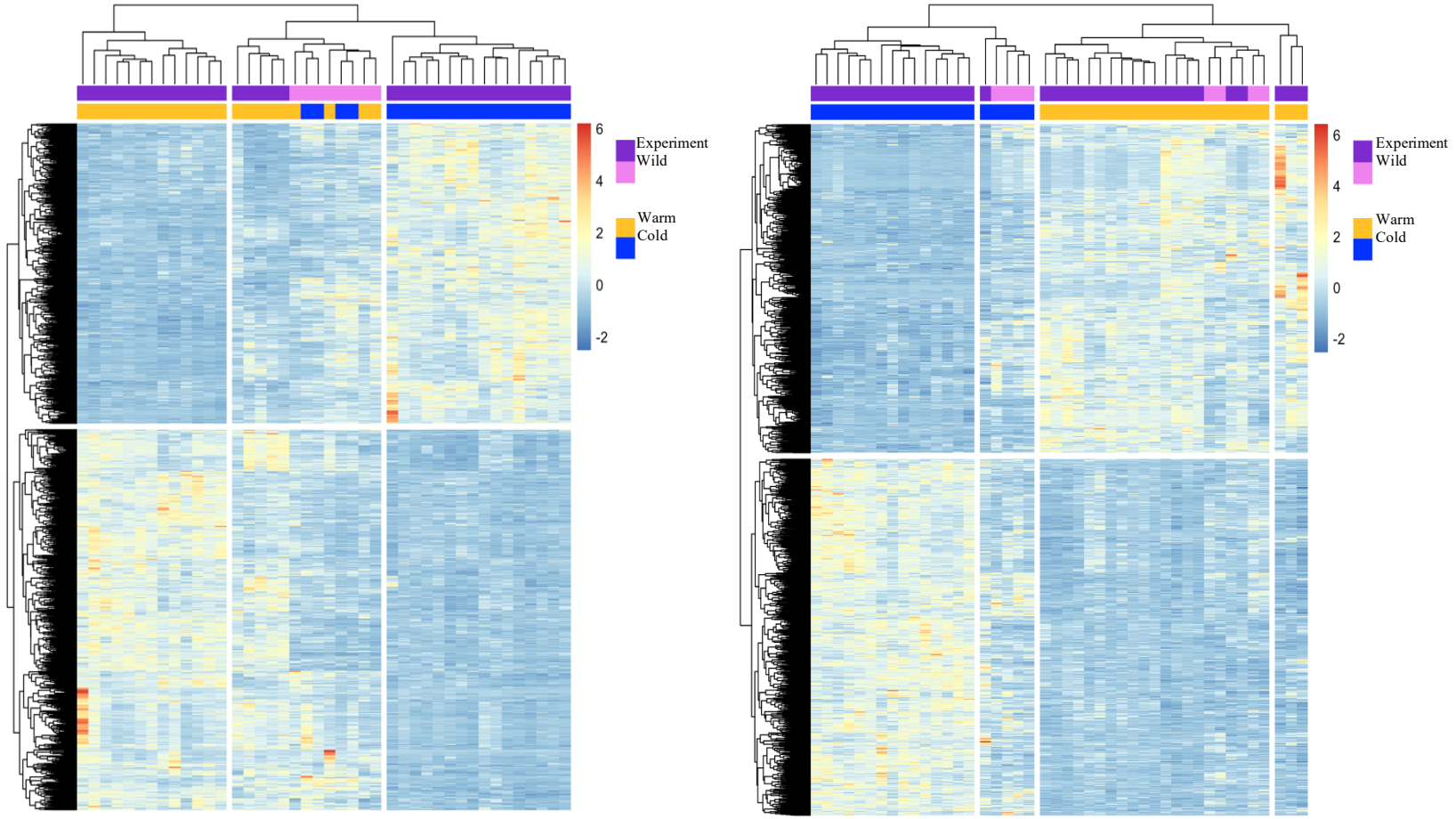

Figure 4.4 *Clustered heat map of standardized Z-scores of differentially expressed genes (adjusted p-value < 0.0001) in livers of 70 lab reared and 16 wild caught threespine stickleback (Gasterosteus aculeatus) in response to contrasting thermal environments. "Experiment" refers to individuals reared under contrasting temperatures (cold:12°C vs. warm:22°C) and "Wild" refers to wild caught individuals from Lake Mývatn, Iceland. "Warm" either refers to warm reared experimental stickleback or wild individuals originating from the "Hot Shore" habitat in Lake Mývatn. "Cold" refers to either cold reared experimental stickleback or wild individuals originating from the "Cold Shore" habitat in Lake Mývatn. A) Gravid females and B) sexually mature males from experimental and wild origin.*

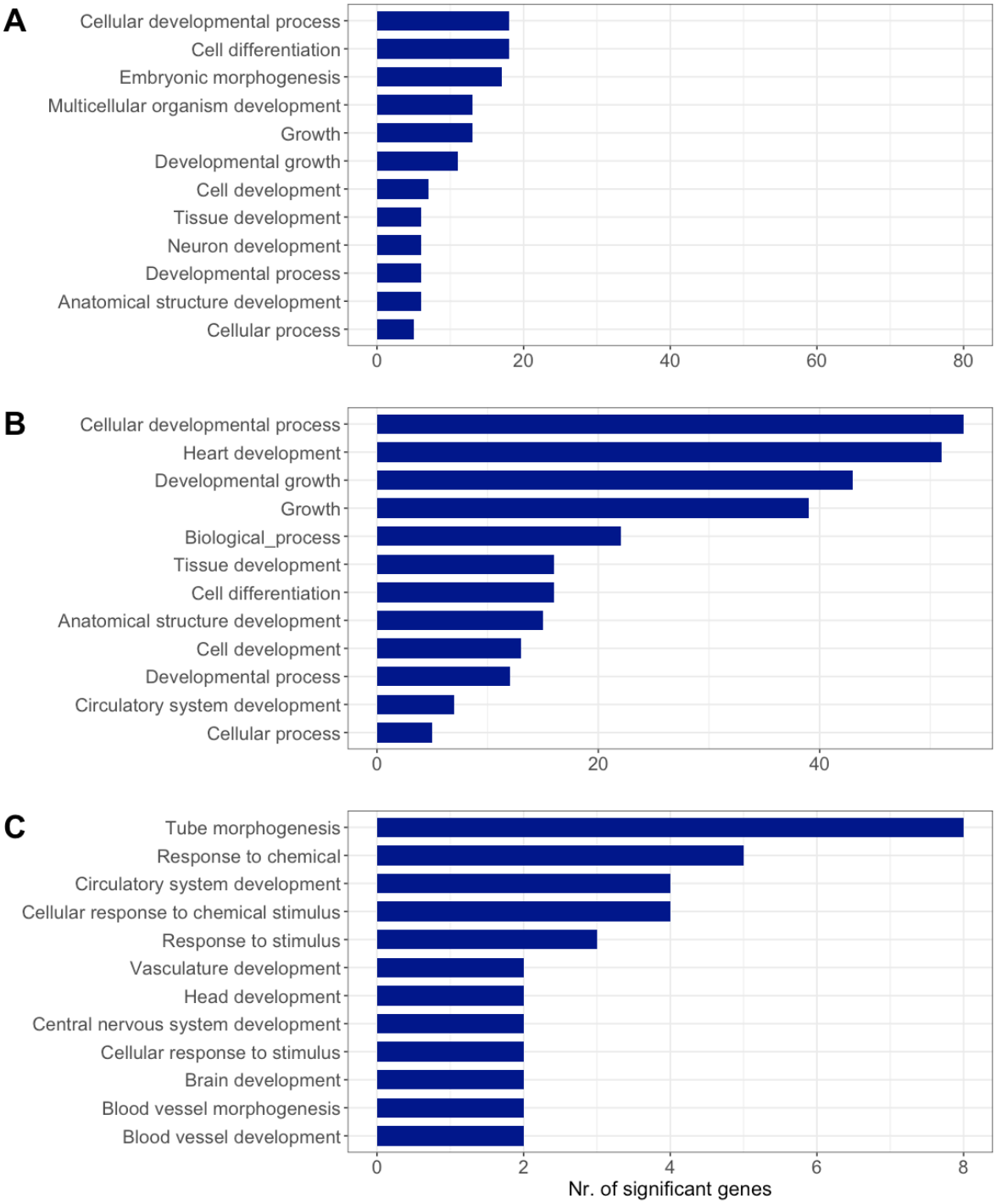

Figure 4.5 *Top 12 gene ontology (GO) terms for differentially expressed genes in livers of first generation threespine stickleback (Gasterosteus aculeatus) reared in a plasticity experiment (see Fig. 2). A) Top 12 GO terms in response to contrasting temperatures (cold:12°C vs. warm:22°C) in A) sexually mature females and B) sexually mature males. C) Top 12 GO terms of sexually mature females in response to contrasting diets (epibenthic vs. benthic).*

data set *aspg* was upregulated in females on an epibenthic diet, suggesting that benthic diet (midge larvae) may be higher in fat.

Furthermore, in females on an epibenthic diet we found upregulated genes mitigating oxidative stress (*sulfoxide reductase B1b: msrb2;* transferrin receptor*: tfr1a*) (Tarifeño-Saldivia et al. 2018; Yin et al. 2024). Contrastingly, *tex264a* is involved in genome stability and DNA replication fork progression (Fielden 2019; Lascaux et al. 2024). Moreover, the *switching B cell complex subunit* (*Swap70*) was downregulated in an epibenthic diet. Swap70 is primarily expressed in B cells, which are crucial to immune response. In teleost fish B cells are generally produced in the thymus and anterior kidney, thus its expression in the liver is likely linked to other functions (Andresen et al. 2024). For example, Swap70 has also been found to be involved in cell signalling and movements (Takada and Appel 2011). Additionally, we found multiple genes traditionally associated with response to heat stress, which were all down regulated on an epibenthic diet (*Hsp70*: *hspa13*, *Hsp40*: *dnajb11*, *dnajc3a*) (He et al. 2023; Liu et al. 2023; Du et al. 2024).

Finally, when looking at the genes that were both differentially expressed in the wild and lab reared individuals, we found additional genes of interest, such as *lipeb*, a lipase that was downregulated in males caught in Hot Shore and males reared in warm temperatures. *lipeb* has been found to be upregulated in liver of rainbow trout (*Oncorhynchus mykiss*) during periods of fasting, as they metabolise fat stored in the liver, until food is available again (Kittilson et al. 2011). Further, *kank1b*, which is involved in cytoskeleton formation, was upregulated in males and females caught in Hot Shore and individuals reared in warm rearing conditions (Hensley et al. 2016). Moreover, in warm reared experimental males and males caught in Hot Shore, the heat shock protein *Hsp90* (*hsp90aa1.2*) was upregulated. Thus, we see comparable patterns in gene expression in response to contrasting environmental temperatures in wild and experimental fish.

4.5 Discussion

Studies investigating gene expression are highly valuable to understand the underlying pathways that produce observed phenotypic variation, and ultimately evolutionary pathways. Furthermore, gene expression patterns are themselves shaped by evolutionary history and, thus, can result in population specific solutions to a common challenge. In this study we investigated potential molecular mechanisms that drive phenotypic divergence in a panmictic population inhabiting contrasting environments, in absence of strong genetic divergence (Strickland et al. 2023). We collected data on differential gene expression (in livers), body size (standard length SL) and allometry (relative head size and body condition (BC)) of threespine stickleback (*Gasterosteus aculeatus*) in response to contrasting temperatures and diets.

We found more extreme differences in expression patterns in experimental individuals, while wild caught individuals displayed more intermediate expression patterns. This is likely due to the lack of confounding environmental variables in experimental individuals; thus, the simplicity of the experimental environment allowed for a clearer signal. We also found clear sexual dimorphism in the gene expression and relative head size. Interestingly, we did not find sexual dimorphism in body size, which is generally well established in this species and population (Kitano et al. 2007; Strickland et al. 2023). Additionally, our results showed that parental origin within lake Mývatn did not strongly affect body size or gene

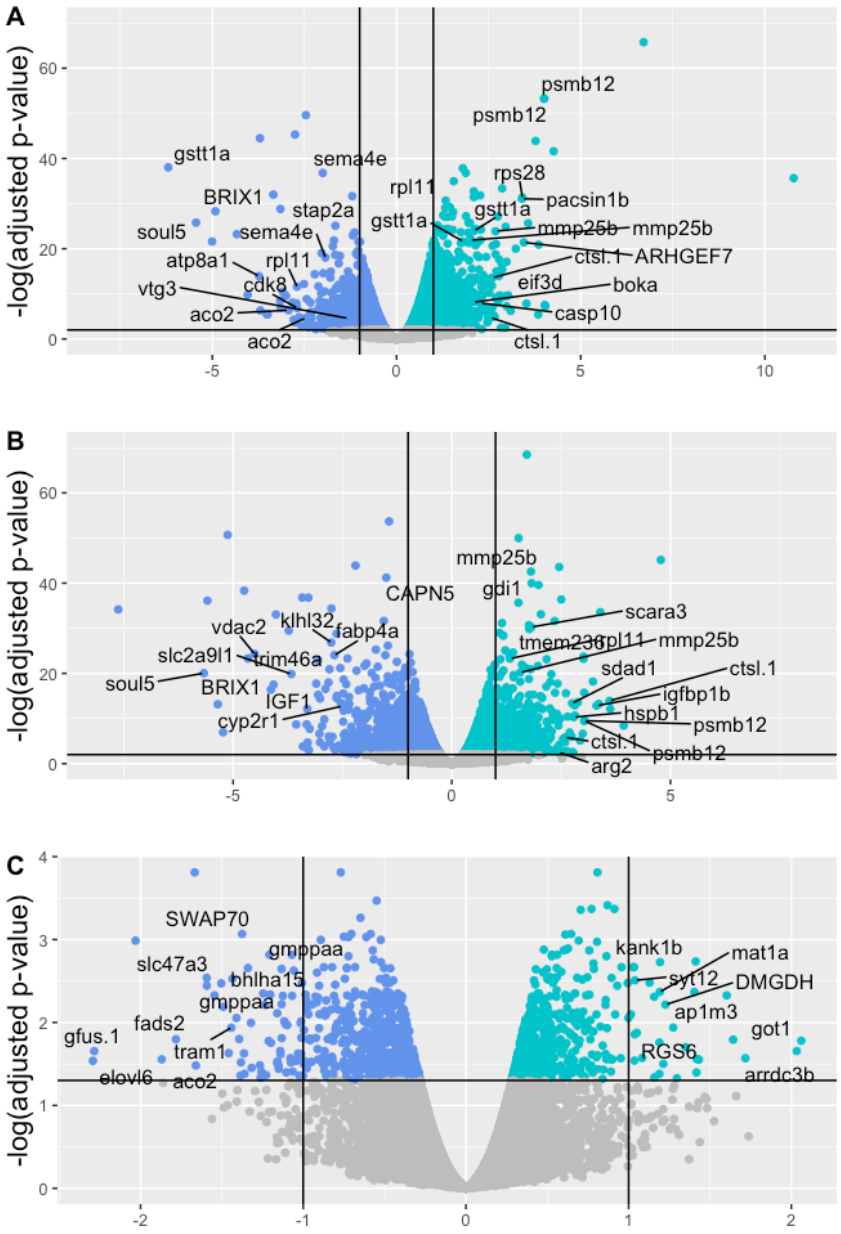

Figure 4.6 *Volcano plot of differentially expressed genes (DEGs) of livers of sexually mature first generation threespine stickleback reared in a plasticity experiment (Fig. 4.1). A) DEGs in gravid threespine stickleback females and B) males reared under contrasting temperatures (cold:12°C vs. warm:22°C). C) DEGs in females reared under contrasting diets (epibenthic vs. benthic). A positive fold change (turquoise) indicates genes upregulated in response to warm temperatures/epibenthic diet whereas negative fold changes (blue) indicate downregulated genes in warm temperatures/epibenthic diet. Genes in grey were below the significance threshold. The horizontal line in A & B marks an adjusted p-value < 0.01 and in C an adjusted p-value < 0.05. Vertical lines indicate a fold change of +1 and -1.*

expression patterns. Thus, we observe a plastic response in gene expression independent of parental origin within the lake. In Mývatn stickleback can potentially encounter highly contrasting habitats, especially regarding temperature, thus, developmental plasticity might play an important role in dispersal.

4.5.1 Effects of temperature on phenotype and growth

We found strong effects of temperature on the phenotype. Threespine stickleback are ectotherms and, therefore, their metabolism is highly correlated with water temperature (Sibly and Atkinson 1994; Ramler et al. 2014). Thus, individuals generally tend to exhibit lower total body size in warm rearing temperatures due to heightened energetic costs that are associated with an increased metabolic rate, which is the opposite of our findings (Angilletta et al. 2004; Ramler et al. 2014). This could reflect that our experimental fish were not limited by resources and thus were able to maximise growth. Indeed, we did supplement all fish with artificial fish pellets that are of high nutritional value. Another explanation could be that fish in the cold rearing conditions simply grow slower, due to a slower metabolism, as indicated by our gene expression data, and thus, fish in the cold rearing temperatures might have grown bigger if given the time. However, in our experiment, the cold-reared fish had ten months to grow, roughly aligning with the age at the start of the breeding season in the wild, when temperature-driven size differences are typically observed.

In the wild population of lake Mývatn, individuals caught in warm temperature habitats are generally shorter than individuals from cold habitats (Millet et al. 2013; Strickland et al. 2023). This supports the hypothesis that fish in warm experimental temperatures were able to maximize growth by removing resource limitation. Nevertheless, we find evidence for warm experimental conditions posing challenges. Fish reared in the warm had bigger relative head size which in other fish has been shown to be a sign of resource limitation, thus resulting in reduced growth of the body whereas the head continues to grow at steady rates, leading to a change in allometry between treatment groups (Post and Parkinson 2001; Olsson et al. 2007). Kotrschal et al. (2012) similarly reported that in Mývatn stickleback, relative male brain size was bigger in the warm habitat compared to the cold habitat, which could indicate that the allometric changes might also have cognitive implications due to contrasting habitat complexity. However, in our experiment habitat complexity was uniform across treatments. Still, it would be interesting to measure brain size in our experimental fish to determine if relative head size does scale with relative brain size.

4.5.2 Maternal investment and reproductive trade-offs

The shift in allometry could also directly impact female fitness, as body size is directly linked to fitness (Wootton 1973; Baker et al. 2015). Females are physically limited in the volume of eggs that they can hold, thus, when resource limitation results in a lower body size, the volume available for reproduction is reduced. Furthermore, females are limited in the energetic investment they can make into the eggs themselves. Vitellogenin is the main precursor protein of egg yolk and thus the main energy source for the developing embryo. Females in the cold, experimental and wild, had higher expression counts of *vtg3* compared to the warm counterparts, indicating that females reared in the cold were investing more energy into reproduction. This is especially interesting as females in the cold produced fewer but bigger eggs (**Paper II**). Together with the transcriptomic data this suggests that females in the cold invested more energy into individual offspring than

females in warm rearing conditions. This could have been due to a higher body condition in cold experimental females, allowing them to invest more energy into their offspring. Alternatively, a longer incubation time in the cold might require more energy for the developing embryo. Similarly, female Amargosa River Pupfish (*Cyprinodon nevadensis amargosae*) in low temperatures had higher vitellogenin transcription levels than females in high temperatures and females experiencing daily fluctuations between high and low temperatures showed intermediate levels of vitellogenin (Housh et al. 2024).

Lipases were generally downregulated in warm rearing conditions, indicating that individuals in cold rearing temperatures stored more fat and thus, had more fat to burn during the energetically costly breeding period. For instance the differentially expressed hormone sensitive lipase b (*lipeb*), which is involved in metabolising fat stored in the liver (Kittilson et al. 2011). Indeed, the highest expression counts of *lipeb* were observed in cold temperature males, which have the highest body condition, whereas warm experimental males showed slightly lower expression and warm wild males exhibited the lowest counts of *lipeb*. This supports our hypothesis that warm reared individuals were not resource limited and thus able to maximise growth, and as part of that, increase energy reserves. It would be interesting to test whether this gene is also upregulated in wild stickleback during the winter months and in other species that undergo periods of recourse limitation, as observed in starved rainbow trout (Kittilson et al. 2011). Furthermore, one could test whether expression levels of *lipeb* are correlated with survival, which would establish this gene as a potential fitness indicator.

4.5.3 Dietary impact on growth and reproduction

Moreover, individuals of both sexes showed lower relative head size and body condition on an epibenthic diet, indicating that benthic prey are a better energy source. Nevertheless, when looking at gene expression in response to contrasting diets we discovered that expression patterns in males were largely unaffected by diet. The differences in male body condition among treatments was also reduced compared to female body condition. Indeed, males in the wild lose weight during breeding season due to increased energy expense during courtship, nest, and brood care, and thus also rely on energy storages (Östlund-Nilsson et al. 2007). This is supported by the increase of *lipeb* in our experimental and wild caught males. Therefore, males with higher fat storage can invest more time and energy into breeding. Consequently, diet appears to be an important factor shaping a male's breeding condition in the run-up to breeding season. This could be especially important in males of species like threespine stickleback, which may reproduce multiple times in one breeding season and do provide parental care (Östlund-Nilsson et al. 2007). Consequently, diet can have a major impact on male reproductive success and ultimately fitness.

When looking at female gene expression patterns in response to diet we found that pathways in relation to chemical and stimulus response and organ development were differentially expressed between diets. However, we also found some genes involved with antioxidant processes to be upregulated and genes in response to heat stress to be downregulated in females fed an epibenthic diet. Thus, an epibenthic diet might help mitigate some of the adverse effects of increased water temperatures. The epibenthic diet had significant fitness implications in our experiment, as individuals showed lower body size, body condition and females produced smaller clutches, which may offer valuable insights into the dynamics of threespine stickleback populations in Lake Mývatn.

4.5.4 Conclusions

Our findings further our understanding of how phenotypic plasticity may be involved in shaping phenotypic variation in a highly dynamic system like the threespine stickleback population of Lake Mývatn. Temperature and diet both had clear effects on phenotype and showed to impact maternal investment and energy allocation. Furthermore, we have found multiple differentially expressed genes in response to temperature and diet that could help mitigate environmental stressors, which would facilitate persistence in challenging environments and dispersal into novel habitats. Finally, many of our findings can most likely be generalized to other systems, however, studies integrating multiple environmental factors across a biologically relevant time frame are still the exception. Our results underscore the importance of such approaches in understanding on how phenotypic plasticity can contribute to biological diversity.

Acknowledgements

Mývatn is a protected area, thus, all sampling was done in collaboration with RAMÝ (The Mývatn Nature Research Station), who holds all permissions to sample in lake Mývatn. Further, permissions from local landowners were obtained to conduct this study on their properties. This study was supported by the Icelandic Research Fund, grant of excellence (195571-052) and doctoral student grant (228501-051). Moreover, we would like to thank all students and technicians that were instrumental in conducting the plasticity experiment. We especially thank Stephen Price who developed and maintained the epibenthic rearing system and assisted with daily management of the experiment. Finally, we thank Kári H. Árnason and Camille A. Leblanc for their invaluable support throughout this project.

References

Adams D, Collyer M, Kaliontzopoulou A, Baken E. 2024. Geomorph: Software for geometric morphometric analyses. https://cran.r-project.org/package=geomorph.

Alexa A, Rahnenfuhrer J. 2024. topGO: Enrichment Analysis for Gene Ontology. https://bioconductor.org/packages/topGO.

Andresen AMS, Taylor RS, Grimholt U, Daniels RR, Sun J, Dobie R, Henderson NC, Martin SAM, Macqueen DJ, Fosse JH. 2024. Mapping the cellular landscape of Atlantic salmon head kidney by single cell and single nucleus transcriptomics. Fish Shellfish Immunol. 146:109357.

Angilletta MJ Jr, Steury TD, Sears MW. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol. 44(6):498–509.

Baken EK, Collyer ML, Kaliontzopoulou A, Adams DC. 2021. geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. Methods Ecol Evol. 12(12):2355–2363.

Baker JA, Wund MA, Heins DC, King RW, Reyes ML, Foster SA. 2015. Life-history plasticity in female threespine stickleback. Heredity . 115(4):322–334.

Bartrons M, Einarsson Á, Nobre RLG, Herren CM, Webert KC, Brucet S, Ólafsdóttir SR, Ives AR. 2015. Spatial patterns reveal strong abiotic and biotic drivers of zooplankton community composition in Lake Mývatn, Iceland. Ecosphere. 6(6):art105.

Bates D, Machler M, Bolker B, Walker SC. 2014. Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 67:1–48.

Benedicenti O, Wang T, Wangkahart E, Milne DJ, Holland JW, Collins C, Secombes CJ. 2017. Characterisation of arginase paralogues in salmonids and their modulation by immune stimulation/ infection. Fish Shellfish Immunol. 61:138–151.

BGI. 2020. [accessed 2020]. https://www.bgi.com/global/.

BioMar SAS. BioMar. [accessed 2024 Feb 20]. https://www.biomar.com/en-gb.

Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Erratum: Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 34(8):888.

Carney Almroth B, Asker N, Wassmur B, Rosengren M, Jutfelt F, Gräns A, Sundell K, Axelsson M, Sturve J. 2015. Warmer water temperature results in oxidative damage in an Antarctic fish, the bald notothen. J Exp Mar Bio Ecol. 468:130–137.

Castro C, Pérez-Jiménez A, Guerreiro I, Peres H, Castro-Cunha M, Oliva-Teles A. 2012. Effects of temperature and dietary protein level on hepatic oxidative status of Senegalese sole juveniles (*Solea senegalensis*). Comp Biochem Physiol A Mol Integr Physiol. 163(3–4):372–378.

Chivers DP, Zhao X, Brown GE, Marchant TA, Ferrari MCO. 2008. Predator-induced changes in morphology of a prey fish: the effects of food level and temporal frequency of predation risk. Evol Ecol. 22(4):561–574.

Chizinski CJ, Pope KL, Wilde GR, Strauss RE. 2010. Implications of stunting on morphology of freshwater fishes. J Fish Biol. 76(3):564–579.

Currey MC, Bassham S, Perry S, Cresko WA. 2017. Developmental timing differences underlie armor loss across threespine stickleback populations. Evol Dev. 19(6):231–243.

Du J, Xie Y, Li M, Zhu T, Lei C, Song H, Han L, Li S. 2024. Effects of chronic heat stress on growth performance, liver histology, digestive enzyme activities, and expressions of HSP genes in different populations of Largemouth bass (*Micropterus salmoides*). Aquaculture Reports. 35:101972.

Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, Huber W. 2005. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics. 21(16):3439–3440.

Durinck S, Spellman PT, Birney E, Huber W. 2009. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nat Protoc. 4(8):1184–1191.

Einarsson Á, Örnólfsdóttir EB. 2004. Long-term changes in benthic Cladocera populations in Lake Myvatn, Iceland. Aquat Ecol. 38(2):253–262.

Einarsson Á, Stefánsdóttir G, Jóhannesson H, Ólafsson JS, Már Gíslason G, Wakana I, Gudbergsson G, Gardarsson A. 2004. The ecology of Lake Myvatn and the River Laxá: Variation in space and time. Aquat Ecol. 38(2):317–348.

Fawcett TW, Frankenhuis WE. 2015. Adaptive explanations for sensitive windows in development. Front Zool. 12 Suppl 1(Suppl 1):S3.

Fielden JC. 2019. TEX264 in DNA replication and genome stability. Ramadan K, editor. University of Oxford.

Fox J, Weisberg S. 2018. An R companion to applied regression. SAGE Publications.

Harrison PW, Amode MR, Austine-Orimoloye O, Azov AG, Barba M, Barnes I, Becker A, Bennett R, Berry A, Bhai J, et al. 2024. Ensembl 2024. Nucleic Acids Res. 52(D1):D891–D899.

He H, Zhang B, Sun S, Liu H, Wang W, Zhou J. 2023. Liver transcriptome analysis revealing response to high-temperature stress in Glyptosternum maculatum (*Sisoridae: Siluriformes*). Aquaculture Reports. 29:101538.

Hensley MR, Cui Z, Chua RFM, Simpson S, Shammas NL, Yang J-Y, Leung YF, Zhang G. 2016. Evolutionary and developmental analysis reveals KANK genes were co-opted for vertebrate vascular development. Sci Rep. 6(1):27816.

Housh MJ, Telish J, Forsgren KL, Lema SC. 2024. Fluctuating and Stable High Temperatures Differentially Affect Reproductive Endocrinology of Female Pupfish. Integr Org Biol. 6(1):obae003.

Ives AR, Einarsson Á, Jansen VAA, Gardarsson A. 2008. High-amplitude fluctuations and alternative dynamical states of midges in Lake Myvatn. Nature. 452(7183):84–87.

Killen SS, Glazier DS, Rezende EL, Clark TD, Atkinson D, Willener AST, Halsey LG. 2016. Ecological Influences and Morphological Correlates of Resting and Maximal Metabolic Rates across Teleost Fish Species. Am Nat. 187(5):592–606.

Kitano J, Mori S, Peichel CL. 2007. Sexual Dimorphism in the External Morphology of the Threespine Stickleback (*Gasterosteus Aculeatus*). Copeia. 2007(2):336–349.

Kittilson JD, Reindl KM, Sheridan MA. 2011. Rainbow trout (*Oncorhynchus mykiss*) possess two hormone-sensitive lipase-encoding mRNAs that are differentially expressed and independently regulated by nutritional state. Comp Biochem Physiol A Mol Integr Physiol. 158(1):52–60.

Kotrschal A, Räsänen K, Kristjánsson BK, Senn M, Kolm N. 2012. Extreme sexual brain size dimorphism in sticklebacks: a consequence of the cognitive challenges of sex and parenting? PLoS One. 7(1):e30055.

Kuparinen A, Cano JM, Loehr J, Herczeg G, Gonda A, Merilä J. 2011. Fish age at maturation is influenced by temperature independently of growth. Oecologia. 167(2):435–443.

Lascaux P, Hoslett G, Tribble S, Trugenberger C, Antičević I, Otten C, Torrecilla I, Koukouravas S, Zhao Y, Yang H, et al. 2024. TEX264 drives selective autophagy of DNA lesions to promote DNA repair and cell survival. Cell. 187(20):5698-5718.e26.

Leblanc CA, Schreck C, Kristjánsson BK, Skúlason S, Noakes DLG. 2023. Egg size–related traits during the first year of growth and smolting in hatchery and wild juveniles of steelhead trout *Oncorhynchus mykiss*. Environ Biol Fishes. 106(5):1061–1078.

Leblanc CA-L, Kristjánsson BK, Skúlason S. 2016. The importance of egg size and egg energy density for early size patterns and performance of Arctic charr *Salvelinus alpinus*. Aquac Res. 47(4):1100–1111.

Li Z, Ndandala CB, Guo Y, Zhou Q, Huang C, Huang H, Li G, Chen H. 2023. Impact of IGF3 stimulation on spotted scat (*Scatophagus argus*) liver: Implications for a role in ovarian development. Agriculture Communications. 1(2):100016.

Liu F, Zhang T, He Y, Zhan W, Xie Q, Lou B. 2023. Integration of transcriptome and proteome analyses reveals the regulation mechanisms of *Larimichthys polyactis* liver exposed to heat stress. Fish Shellfish Immunol. 135:108704.

Lubzens E, Bobe J, Young G, Sullivan CV. 2017. Maternal investment in fish oocytes and eggs: The molecular cargo and its contributions to fertility and early development. Aquaculture. 472:107–143.

Matsuda T, Oritani K. 2021. STAP-2 adaptor protein regulates multiple steps of immune and inflammatory responses. Biol Pharm Bull. 44(7):895–901.

McGee MD, Schluter D, Wainwright PC. 2013. Functional basis of ecological divergence in sympatric stickleback. BMC Evol Biol. 13:277.

Méndez L, Ciordia S, Fernández MS, Juárez S, Ramos A, Pazos M, Gallardo JM, Torres JL, Nogués MR, Medina I. 2017. Changes in liver proteins of rats fed standard and high-fat and sucrose diets induced by fish omega-3 PUFAs and their combination with grape polyphenols according to quantitative proteomics. J Nutr Biochem. 41:84–97.

Millet A. 2013. Spatio-temporal variation of threespine stickleback (*Gasterosteus aculeatus*) in the dynamic Lake Mývatn, Iceland. Kristjánsson BK, Räsänen K, editors. PhD thesis, University of Iceland.

Millet A, Kristjánsson BK, Einarsson Á, Räsänen K. 2013. Spatial phenotypic and genetic structure of threespine stickleback (*Gasterosteus aculeatus*) in a heterogeneous natural system, Lake Mývatn, Iceland. Ecol Evol. 3(10):3219–3232.

Nash RDM, Valencia AH, Geffen AJ. 2006. The origin of Fulton's condition factor—setting the record straight. Fisheries. 31(5):236–238.

Nath S, Shaw DE, White M. 2020. Improved contiguity of the threespine stickleback genome using long-read sequencing. G3: Genes|Genomes|Genetics. 11.

Olsson J, Svanbäck R, Eklöv P. 2007. Effects of resource level and habitat type on behavioral and morphological plasticity in Eurasian perch. Oecologia. 152(1):48–56.

Oomen RA, Hutchings JA. 2017. Transcriptomic responses to environmental change in fishes: Insights from RNA sequencing. Facets (Ott). 2(2):610–641.

Östlund-Nilsson S, Mayer I, Huntingford F. 2007. Reproductive behaviour in the three-spined stickleback. In: Biology of the three-spined stickleback. CRC Press. p. 157–178.

Pédron N, Artigaud S, Infante J-LZ, Le Bayon N, Charrier G, Pichereau V, Laroche J. 2017. Proteomic responses of European flounder to temperature and hypoxia as interacting stressors: Differential sensitivities of populations. Sci Total Environ. 586:890–899.

Pfennig DW. 2021. Phenotypic Plasticity & Evolution: causes, consequences, controversies. Taylor & Francis (Evolutionary Cell Biology).

Phillips JS, Einarsson Á, Strickland K, Ives AR, Kristjánsson BK, Räsänen K. 2023. Demographic Basis of Spatially Structured Fluctuations in a Threespine Stickleback Metapopulation. Am Nat. 201(3):E41–E55.

Pimentel H, Bray NL, Puente S, Melsted P, Pachter L. 2017. Differential analysis of RNA-seq incorporating quantification uncertainty. Nat Methods. 14(7):687–690.

Pimentel H MW. 2024. sleuth: Tools for investigating RNA-Seq. https://github.com/pachterlab/sleuth.

Posit team. 2023. RStudio: Integrated Development for R. http://www.posit.co/.

Post JR, Parkinson EA. 2001. Energy allocation strategy in young fish: Allometry and survival. Ecology. 82(4):1040.

R Core Team. 2023. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Ramler D, Mitteroecker P, Shama LNS, Wegner KM, Ahnelt H. 2014. Nonlinear effects of temperature on body form and developmental canalization in the threespine stickleback. J Evol Biol. 27(3):497–507.

Shama LNS, Strobel A, Mark FC, Wegner KM. 2014. Transgenerational plasticity in marine sticklebacks: maternal effects mediate impacts of a warming ocean. Funct Ecol. 28(6):1482–1493.

Sibly RM, Atkinson D. 1994. How Rearing Temperature Affects Optimal Adult Size in Ectotherms. Funct Ecol. 8(4):486–493.

Singaraja RR, James ER, Crim J, Visscher H, Chatterjee A, Hayden MR. 2005. Alternate transcripts expressed in response to diet reflect tissue-specific regulation of ABCA1. J Lipid Res. 46(10):2061–2071.

Skúlason S, Parsons KJ, Svanbäck R, Räsänen K, Ferguson MM, Adams CE, Amundsen P-A, Bartels P, Bean CW, Boughman JW, et al. 2019. A way forward with eco evo devo: an extended theory of resource polymorphism with postglacial fishes as model systems. Biol Rev Camb Philos Soc. 94(5):1786–1808.

Snorradóttir RG. 2023. Relationships between phenotype, diet, and individual specialization of threespine stickleback. Kristjánsson BK, editor. MSc thesis, Hólar University.

Strickland K, Matthews B, Jónsson ZO, Kristjánsson BK, Phillips JS, Einarsson Á, Räsänen K. 2024. Microevolutionary change in wild stickleback: Using integrative time-series data to infer responses to selection. Proc Natl Acad Sci USA. 121(37):e2410324121.

Strickland K, Räsänen K, Kristjánsson BK, Phillips JS, Einarsson Á, Snorradóttir RG, Bartrons M, Jónsson ZO. 2023. Genome-phenotype-environment associations identify signatures of selection in a panmictic population of threespine stickleback. Mol Ecol. 32(7):1708–1725.

Sultan SE, Spencer HG. 2002. Metapopulation structure favors plasticity over local adaptation. Am Nat. 160(2):271–283.

Takada N, Appel B. 2011. Swap70 promotes neural precursor cell cycle exit and oligodendrocyte formation. Mol Cell Neurosci. 48(3):225–235.

Tarifeño-Saldivia E, Aguilar A, Contreras D, Mercado L, Morales-Lange B, Márquez K, Henríquez A, Riquelme-Vidal C, Boltana S. 2018. Iron overload is associated with oxidative stress and nutritional immunity during viral infection in fish. Front Immunol. 9:1296.

Taugbøl A, Junge C, Quinn TP, Herland A, Vøllestad LA. 2014. Genetic and morphometric divergence in threespine stickleback in the Chignik catchment, Alaska. Ecol Evol. 4(2):144–156.

West-Eberhard MJ. 2003. Developmental Plasticity and Evolution. Oxford University Press.

Wootton RJ. 1973. Fecundity of the three-spined stickleback, *Gasterosteus aculeatus* (L.). J Fish Biol. 5(6):683–688.

Xie D, Chen C, Dong Y, You C, Wang S, Monroig Ó, Tocher DR, Li Y. 2021. Regulation of long-chain polyunsaturated fatty acid biosynthesis in teleost fish. Prog Lipid Res. 82(101095):101095.

Yilmaz O, Sullivan CV, Bobe J, Norberg B. 2024. The role of multiple vitellogenins in early development of fishes. Gen Comp Endocrinol. 351(114479):114479.

Yin P, Saito T, Fjelldal PG, Björnsson BT, Remø SC, Sharma S, Olsen RE, Hamre K. 2024. Environmentally driven changes in Atlantic salmon oxidative status interact with physiological performance. Aquaculture. 581:740400.

Appendix A: Supplementary Paper I

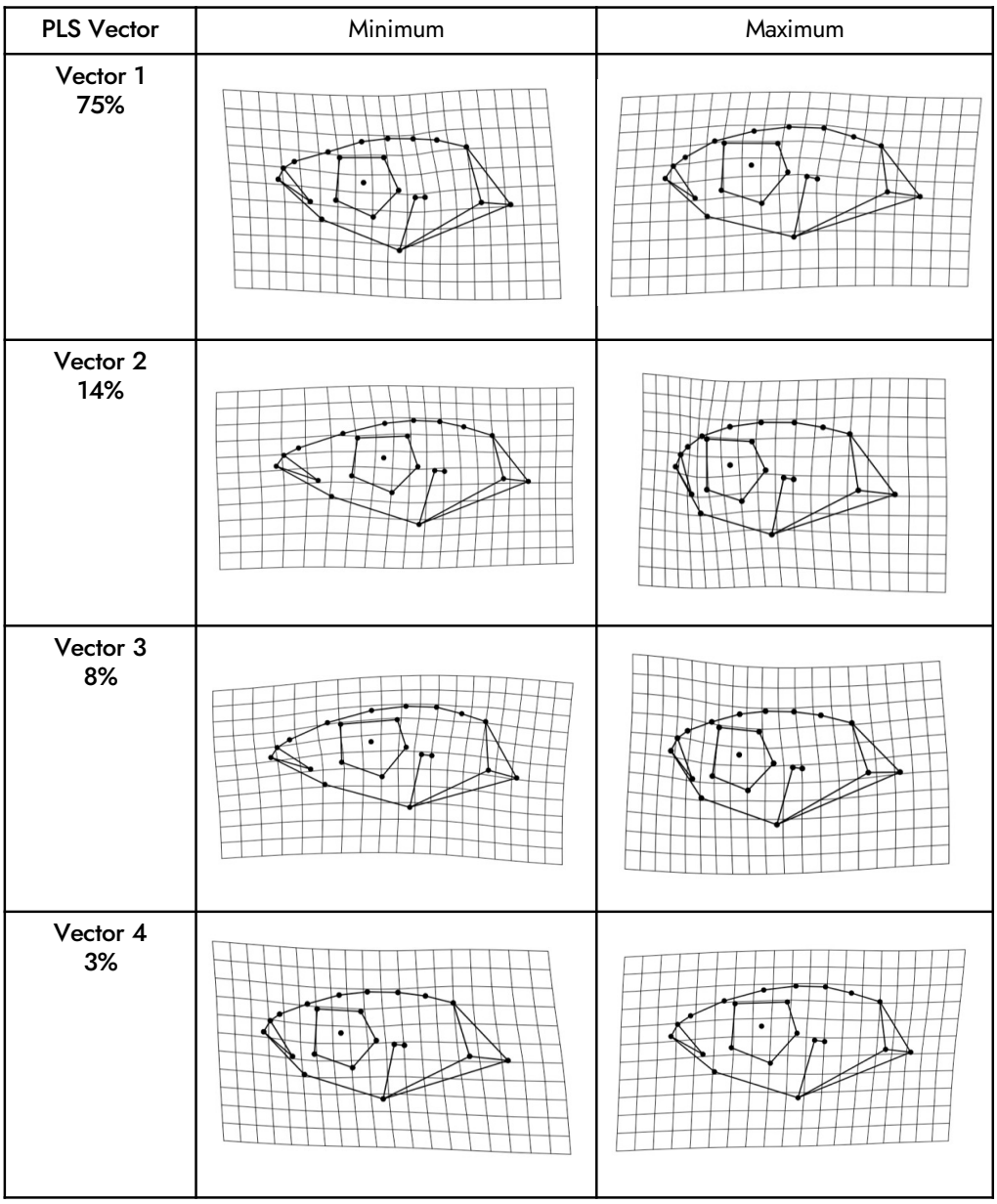

Figure A.1 *Deformation grids represent the shape at the extreme ends of PLS1 – PLS4 axis. The deformation grids were plotted with a 1 magnification. Percentages indicate how much variation each vector explains. The first vector explains the majority of the variation. The minimum head shape has a more elongated head shape, whereas the maximum shows a more rounded and shorter head shape with a relatively larger eye. The minimum is associated with a Chironomidae larvae whereas the maximum is associated with a Cladocera diet. The minimum of vector 2 and 3 have both an elongated head shape and pointed mouth whereas the maxima have a stunted snout and an upturned mouth. The minimum of vector 2 is associated with a diet consisting of small Cladocera and Chironomidae larvae, whereas the maximum is associated with big Cladocera and other various diet items. The minimum of the third vector is associate with big Cladocera and the minimum with adult Chironomidae and various diet items. The minimum of the 4th vector, which is associated with feeding on adult Chironomidae, shows an upturned mouth and an elongated opercular region compared to the maximum, which is a associated with a more mixed diet on various diet items.*

Appendix B: Supplementary Paper II

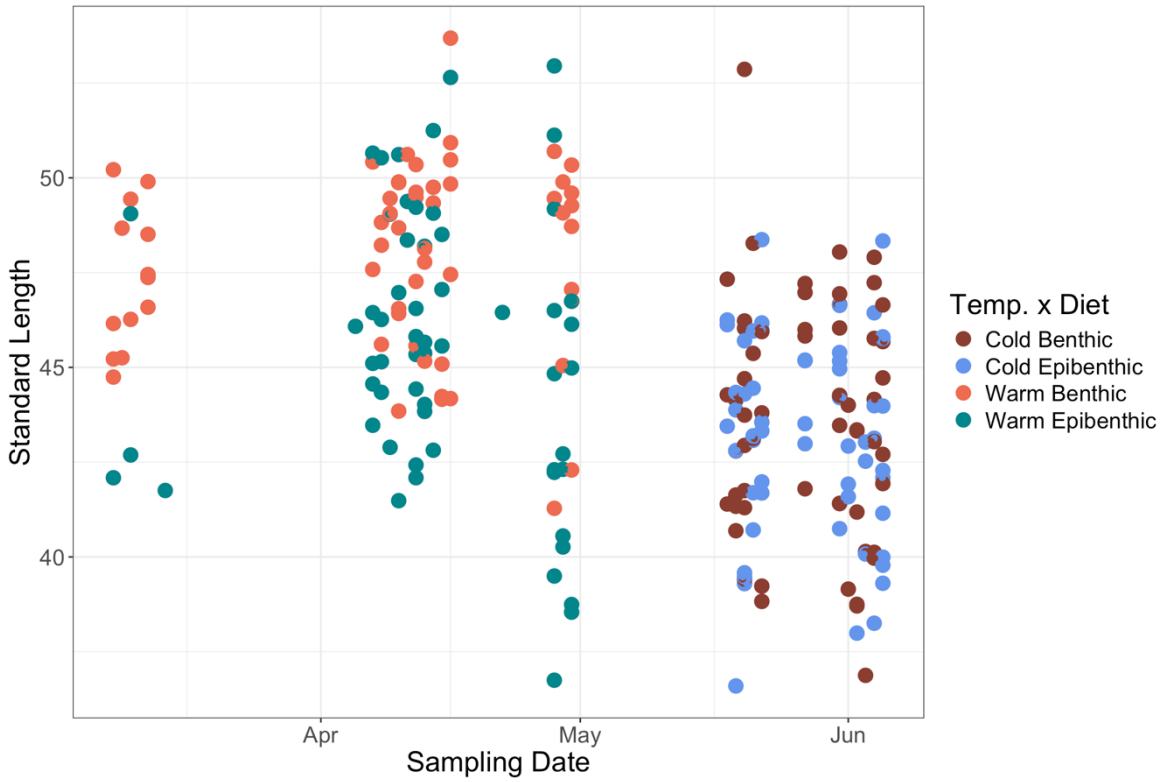

Figure B.1 *Standard body length (mm) of sexually mature F1 female threespine stickleback reared in a transgenerational plasticity experiment under contrasting temperatures (cold:12°C vs. warm:22°C) and diets (benthic (midge larvae) vs. epibenthic (Cladocera)) (see Fig. 3.2). Sampling date refers to the day the females were sampled and photographed. Standard length was measured from these photographs taken on sampling day (see 3.7.7). Individual points represent uncorrected measurements of standard body length (mm) of individual F1 females.*

Appendix C: Supplementary Paper III

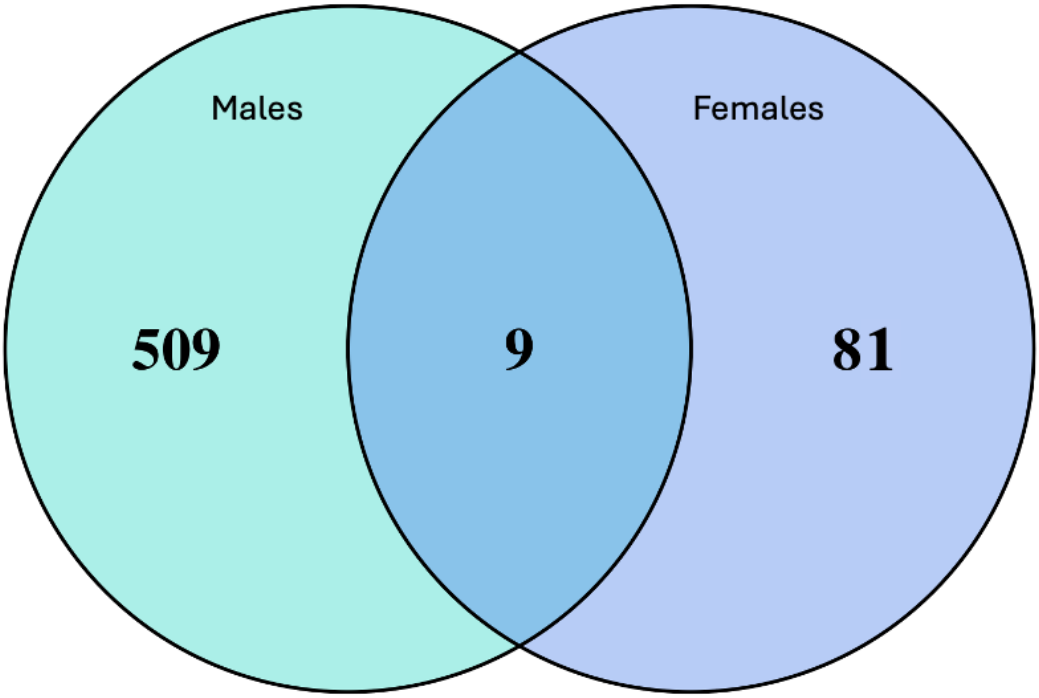

Figure C.1 *Number of differentially expressed genes in livers of wild caught male and female threespine stickleback (Gasterosteus aculeatus) from two contrasting habitats within Lake Mývatn, Iceland, Cold Shore and Hot Shore (see Fig. 3.1).*

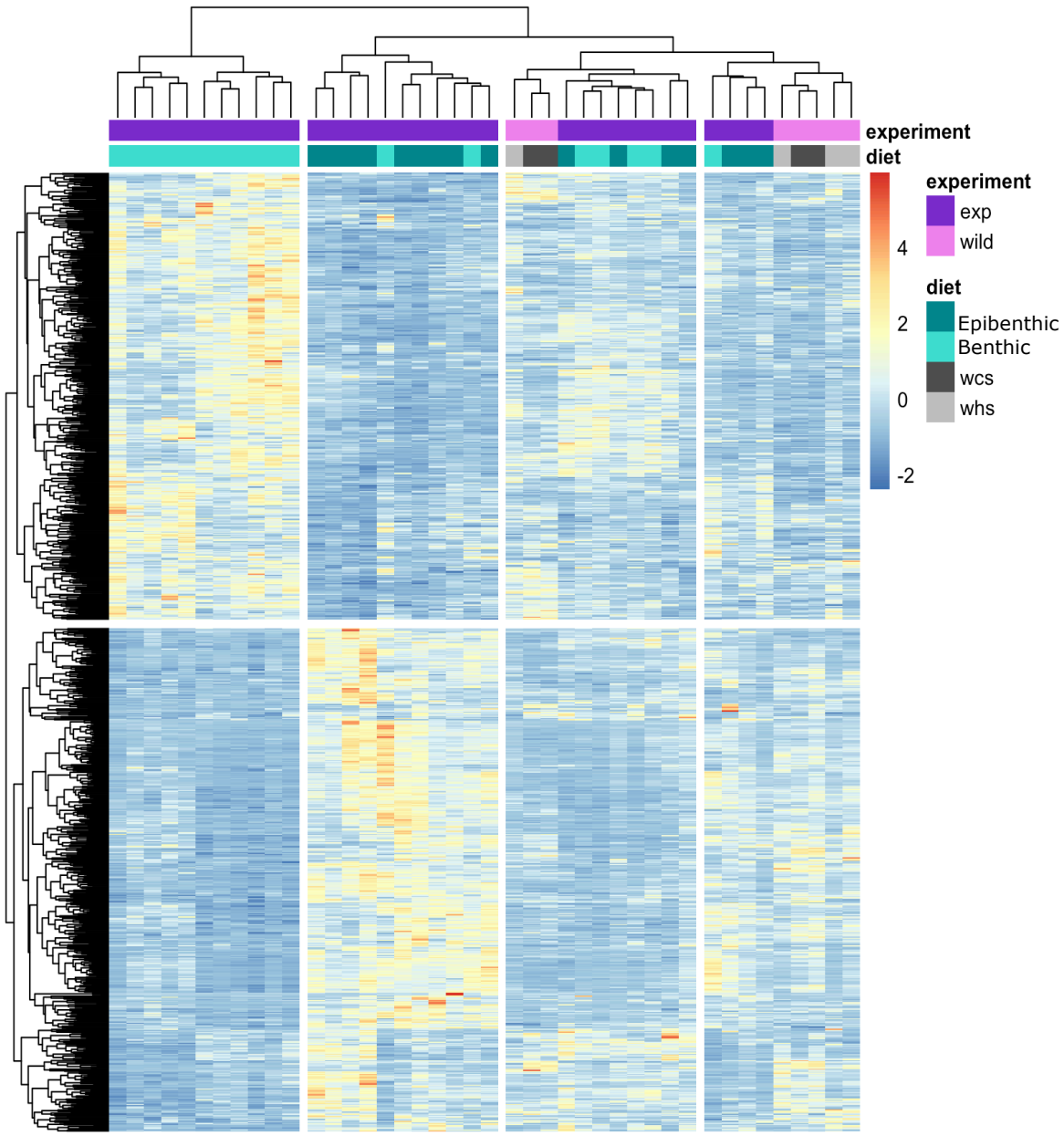

Figure C.2 *Clustered heat map of standardized Z-scores of differentially expressed genes (adjusted p-value < 0.0001) in livers of 34 lab reared and 8 wild caught female threespine stickleback (Gasterosteus aculeatus) in response to contrasting environments. Experiment refers to individuals either originating from a plasticity experiment (exp) where individuals were reared under contrasting diets (epibenthic: Cladocera vs. benthic: midge larvae) or wild caught individuals from Lake Mývatn, Iceland (wcs = Wild Cold Shore, whs = Wild Hot Shore) (see Fig. 3.1).*