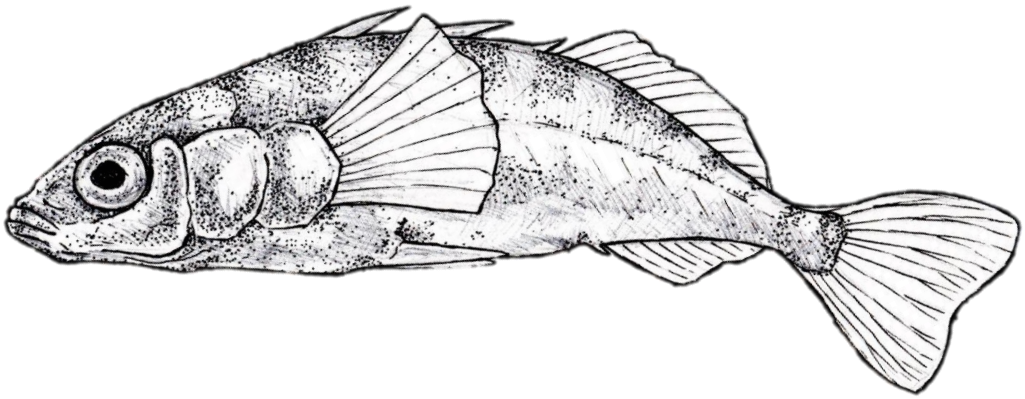
Evolution and Plasticity in Geothermal Three-spined Sticklebacks (*Gasterosteus aculeatus*)

By

Bethany Smith



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Abstract

Ectotherms are expected to be particularly vulnerable to climate change driven increases in temperature. Understanding how populations adapt to novel thermal environments will be key for informing mitigation plans. I took advantage of threespine stickleback (*Gasterosteus aculeatus*) populations inhabiting adjacent geothermal and ambient habitats to test for evidence of adaptive divergence and plasticity.

In the first data chapter, Chapter 2, I performed a reciprocal transplant experiment to assess the effects of transplantation and morphological variation on growth and survival. I found evidence for adaptive morphological divergence, as growth (length change) in the non-native habitat was found to relate to head, posterior and total body shape. Higher growth in fish transplanted to a non-native habitat was found to be associated with shape profiles closer to that of the native fish. The consequences of transplantation were asymmetric with ambient sourced fish transplanted to the geothermal habitat suffering from lower survival rates and greater parasite prevalence than geothermal sourced fish transplanted to the ambient habitat. I also found evidence for divergent shape allometries that related to growth. My findings suggest that wild populations can adapt quickly to thermal conditions. However, immediate transitions to warmer conditions may be particularly difficult.

Whole transcriptome gene expression can provide a wholly objective insight into divergence and plasticity in a population. In the second data chapter, Chapter 3, I used an F1 generation of geothermal and ambient sticklebacks reared at 12°C and 18°C to test for evidence of robust divergence in gene expression and divergence in plasticity. I also used F1 hybrids, generated from crossing geothermal and ambient sticklebacks, to assess the genomic and regulatory mechanisms behind geothermal-ambient divergence. I found a small number of genes related to neuron development and functioning to be downregulated in the brains of geothermal fish at both rearing temperatures. Additionally, I found a large number of genes to be plastic in the brains of geothermal fish, largely relating to metabolism. I found little evidence of additive variation or cis-regulatory divergence, suggesting that there is little evolutionary divergence between geothermal and ambient fish. However, I found evidence of potential reproductive isolation between geothermal and ambient

sticklebacks in the form of a high degree of transgressive expression in hybrid fish.

In my final data chapter, Chapter 4, I assessed for consistent divergence in gene expression and adaptive plasticity across three population pairs of geothermal and ambient sticklebacks. I found evidence of consistent divergence across two populations, largely in genes relating to glucose metabolism. I also found evidence of a consistent divergent plastic response in geothermal sticklebacks involving an upregulation in genes relating to the innate and adaptive immune system.

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Ethics statement

All work conducted in this study adhered to the ASAB/ABS Guidelines for the Use of Animals in Research, the institutional guidelines at University of Glasgow, and the legal requirements of the UK Home Office (Project License P89482164). In Iceland, permissions were obtained from local landowners for sampling and experimental set up. All animal storing and handling was conducted under the animal care license (FE-1051) of the Hólar University Research Station, Verið, issued by the Icelandic food and veterinary authority.

Authors declaration

The research conducted in this thesis took place between October 2018 and September 2023 and is entirely my own, none of the work in this thesis has been submitted for another degree.

Bethany Smith,

October 2023

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List of Abbreviations

AB	Ambient brain
AL	Ambient liver
Amb.	Ambient
ASHN	Áshildarholtsvatn population pair
DE	Differential expression
GB	Geothermal brain
GE	Gene expression
Geo.	Geothermal
GL	Geothermal liver
GLM	Generalized linear model
HB	Hybrid brain
HL	Hybrid liver
MYV	Mývatn
PCA	Principal component analysis
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SKR	Sauðárkrókur population pair
SNP	Single nucleotide polymorphism
WGCNA	Weighted gene co-expression network analysis

1 Chapter 1: General introduction

1.1 The threat of climate change

The ability for man-made greenhouse gases to alter the climate was first recognised in the late 19th century by Swedish scientist Svante Arrhenius. Initially suggested to be a potentially beneficial side effect, it is now recognised that anthropogenic climate change is one of the greatest threats to global biodiversity in human history. However, progress has been slow in understanding and addressing the ramifications of this phenomenon. Between 1880 and 2012, an increase of 0.85°C has been observed in the global mean surface temperature (GMST) (IPCC, 2013). Furthermore, the rate of temperature increase has itself been accelerating, with the per decade temperature change from 1979 to 2012 around 50% greater than 1901 to 1950 (IPCC, 2013). In the Intergovernmental Panel on Climate Change's (IPCC) fifth assessment report (2013) climate modelling predicts that trends of increasing surface temperature caused by increasing levels of greenhouse gases will continue. Representative concentration pathway (RCP) models project near-future (2016-2035) global temperature changes of between 0.3°C and 0.7°C relative to 1986-2005, and far-future (2081–2100) global temperature changes of between 0.3°C and 4.8°C, with the severity dependent on emission levels (IPCC, 2013). Current goals set by the IPCC are to limit GMST warming to well below 2°C above pre-industrial temperatures, with below 1.5°C preferred. However, the effects of even a mere 1.5°C change in global temperature are likely to be vast (IPCC, 2013, 2018). Furthermore, while there may only be a 1.5°C change in GMST, certain regions of the world will face greater changes in temperature than others, and some have already experienced temperature changes greater than 1.5°C (IPCC, 2013). Arctic regions are likely to suffer from the greatest changes in temperature, with low emission models suggesting an increase of 2.2°C and high emission models suggesting an increase of up to 8.3°C by 2081-2100 (IPCC, 2013). Additionally, anthropogenic warming is predicted to occur at a greater rate over land areas, and so freshwater habitats will face greater, and faster, temperature changes than many marine habitats (IPCC, 2013). Ultimately, animal populations around the world face a severe threat from climate change. Some populations may be more vulnerable than others due to unique aspects of their habitat and biology. It is expected that these animal populations will need to adapt and change in response to these threats.

1.1.1 Evolvability and phenotypic plasticity

Once thought to always be a long and slow process, it is now known that evolutionary adaptation can, at least in some cases, occur rapidly (Hendry et al., 2008). As such, the capacity for a population of organisms to evolve in the face of climate change is a key concern in developing effective conservation management plans for the future (Campbell et al., 2017; Hansen et al., 2012; Hoffmann and Sgró, 2011; Kokko et al., 2017).

The importance of total genetic diversity in evolution has been recognised for some time, but exactly how much of this genetic diversity contributes to evolutionary potential is more difficult to assess (Reed and Frankham, 2001; Whitlock, 2014). For example, Meffe et al (1995) determined that genetic variation (in the form of heterozygosity) was key to the survival of eastern mosquitofish (*Gambusia holbrooki*) under high temperatures, but Kavanagh et al (2010) found that grayling demes were able to locally adapt to thermal habitats despite low genetic diversity and significant gene flow. High total genetic variation does not necessarily reflect the levels of genetic variation in traits under selection (Hoffmann et al., 2003). Furthermore, considering only total genetic variation does not capture the potential for interactions between genes and the environment in phenotypic plasticity (Campbell et al., 2017; Kokko et al., 2017).

The concept of evolvability has been used as a framework that goes beyond total genetic variation in its description of evolutionary potential (Campbell et al., 2017; Feiner et al., 2021; Parsons, 2021). The evolvability framework considers the adaptive potential of genetic variation and the interactions between genotype, phenotype and environment (Campbell et al., 2017). Phenotypic plasticity is the ability of a single genotype, or developmental system, to give rise to different phenotypes depending on the environmental conditions experienced (Bradshaw, 1965; Parsons, 2021). Phenotypic plasticity can interact with the evolutionary process in a myriad of ways.

Perhaps the simplest way in which phenotypic plasticity can contribute to evolution is by “buying time” for evolutionary change to occur. Organisms faced with changing environments or colonising a novel habitat are faced with immediate stresses that may prevent survival or reproduction, without which, evolutionary change cannot occur. The “Baldwin effect” proposes that plastic

responses may allow for populations to shift their phenotype in line with these new selective pressures, providing them with the means to survive (Baldwin, 1896; Crispo, 2007). Furthermore, these initial plastic responses may be able to guide the evolutionary process through "plasticity-led evolution" (Levis and Pfennig, 2016, 2019). The initial adaptive, but suboptimal, plastic responses may themselves be refined and canalised through genetic changes over time (Levis and Pfennig, 20(Crispo, 2007; Waddington, 1953)16, 2019). Plastic responses can also aid the evolutionary process by revealing cryptic genetic variation to selection (Parsons et al., 2020). However, plasticity is also able to hinder evolution. Phenotypic plasticity can mask genetic variation from selective pressures and facilitate gene flow, potentially preventing local adaptation (Crispo, 2008; Ghalambor et al., 2015). Furthermore, the high potential for non-adaptive plasticity, where plastic responses do not improve fitness, suggests that there may be an advantage to being phenotypically robust (Uller et al., 2013). However, non-adaptive plasticity has been found to potentially aid evolution by strengthening selective pressures (Ghalambor et al., 2015). Additionally, the extent to which phenotypic plasticity can compensate for environmental change is limited, and may be compromised by complicated interactions between stressors (Both, 2010). Ultimately, the interactions between phenotypic plasticity and evolution are complex.

1.1.2 Climate change and fish

Temperature is a fundamental aspect of biology, affecting processes from the scale of the molecular to the ecological (Brown et al., 2004). The temperature of the environment has an especially strong effect on ectotherms, which are limited in their ability to regulate their internal temperature (Brown et al., 2004; Fry, 1967; Paaijmans et al., 2013). Fish, most of which are ectotherms, are therefore expected to be vulnerable to the effects of climate change (Fry, 1967). Freshwater fish in particular, face faster and greater increases in temperature than fish in many marine habitats (IPCC, 2013, 2021). Freshwater fish often have limited dispersal options and so may not be able to escape increased temperatures through range shifts (Dudgeon et al., 2006).

The organism level effects of increased temperature have been well documented in fish, with cell level processes resulting in an increased metabolic rate generating a greater demand for oxygen (Clarke and Johnston, 1999; Fry,

1967). The survival and performance of fish under acute warming is reliant on their ability to supply enough oxygen to meet this increased demand (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). Failure to adapt or acclimate to acute warming or to long term chronic warming would be expected to result in extinction or local extirpation. Longer term, chronic effects may also occur even when the individual fish appears to have acclimated sufficiently for survival and reproduction (Pörtner and Farrell, 2008). An increased metabolic rate induced by increased temperatures would be expected to increase nutritional needs and alter growth rates, and distribution changes would be expected with shifting temperature ranges and altered prey distribution (Helaouët and Beaugrand, 2009).

Population changes in fish are already being observed in response to climate change, including shifts in distribution (Brander et al., 2003; Grebmeier et al., 2006; Perry et al., 2005), changes in migratory behaviour (Farrell et al., 2008) and even local extinctions (Becker et al., 2018; Pörtner and Knust, 2007). Many of these shifts in phenology have been found to be plastic in nature, including alterations in migration timing, (Crozier et al., 2011; Dahl et al., 2004; Juanes et al., 2004; Kennedy and Crozier, 2010; Quinn and Adams, 1996; Sims et al., 2013), spawn timing (Gillet and Quétin, 2006; Schneider and Newman, 2010), growth (Friedland et al., 2005; Hurst et al., 2012; Teal et al., 2008; Todd et al., 2008), size and age at maturity (Attrill and Power, 2002; Cox and Hinch, 1997; Jonsson and Jonsson, 2004; Otero et al., 2012; Ottersen et al., 2006; Rogers et al., 2011; Schindler et al., 2005), fecundity (Nakken, 2005) and juvenile survival (Beaugrand et al., 2003). Meanwhile, relatively few cases of evolutionary responses to climate change have been verified in fish. Climate change driven genetic shifts have been found to be involved in the altered migration timing of pink salmon (*Oncorhynchus gorbuscha*) (Kovach et al., 2012) and sockeye salmon (*Oncorhynchus nerka*) (Crozier et al., 2011).

1.2 Thermal adaptation and plasticity in fish

As temperature affects such a wide array of biological processes, adaptation and plastic responses to increased temperature are varied and complex. Research investigating the potential routes of adaptive responses to climate change can utilise several different approached. Laboratory based experiments exploring the responses of fish to acute warming can reveal the

initial, plastic responses of fish. Many fish are able to acclimate to temperatures somewhat beyond their optimal range in a plastic response involving widespread physiological changes (Lagerspetz, 2006). Acclimation can be either a reversible response to a temporary fluctuation, or a permanent response induced by the environment during ontogeny (developmental acclimation) (Angilletta, 2009). Thermal acclimation permits survival under increased temperatures, however, the degree to which a fish can acclimate varies greatly by species, leaving many vulnerable to climate change (Donelson, 2015; Peck et al., 2014; Tomanek, 2010).

Insights into the potential for long-term adaptation to warmed habitats can be gained by taking advantage of thermal gradients occurring in the wild. Many fish species occur over wide spatial distances, and so naturally encounter thermal gradients, whether due to latitude or more complex aspects of geography. Investigations into local adaptation along these thermal gradients can reveal the mechanism behind thermal adaptation (Baumann and Conover, 2011; Kim et al., 2017). Short distance gradients may also be found, caused by geothermal springs or man-made hot water effluents. Short distance gradients are especially promising, as they allow for the investigation of temperature without confounding factors such as latitude, altitude, habitat composition or photoperiod (Gallo et al., 2017; O'Gorman et al., 2014). The young age of many man-made hot water effluents also provides a valuable opportunity to observe the early stages of thermal adaptation (Dayan et al., 2019).

Thermal adaptation may involve changes to metabolism and physiology (Jutfelt, 2020). Metabolic rate increases with temperature in fish, increasing the energy required for basic maintenance and reducing their aerobic scope, the ability of an animal to raise its metabolic rate above basic maintenance (Fry, 1967). As sufficient aerobic scope is necessary for an animal to invest energy into activity and growth, this presents a strong selective pressure. Indeed, fish from different thermal habitats have been found to show adaptive divergence in growth and reproduction, potentially as a consequence of these pressures (Kavanagh et al., 2010). Fish from warmed habitats tend to show a higher critical thermal maximum than conspecifics from ambient temperature habitats (Chen et al., 2018; Garvin et al., 2015; Meffe et al., 1995). This can be a result of a variety of changes in metabolism and physiology that allow warm-adapted

fish to increase their aerobic scope at higher temperatures. For example, redband trout (*Oncorhynchus mykiss gairdneri*) from desert habitats have been found to exhibit alterations to heart rate and the expression of genes relating to mitochondrial function (Chen et al., 2018; Garvin et al., 2015). Three-spined sticklebacks inhabiting geothermal habitats exhibit lower metabolic rates than sticklebacks from ambient temperature habitats when kept at a common temperature (Pilakouta et al., 2020). Evidence of adaptation in metabolic rate to temperature has been found in a number of species of fish (Eliason et al., 2011; Healy and Schulte, 2012; Lucassen et al., 2006; Di Santo, 2016; Sylvestre et al., 2007). Additionally, oxygen demand increases with metabolic rate, and so acute warming can put fish at risk of hypoxia (McBryan et al., 2013; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2005). Improved tolerance of increased temperature has been found to relate to hypoxia tolerance in fish (Anttila et al., 2013; Firth et al., 2023).

Morphological divergence has also been observed in response to thermal habitats. The body morphology of a fish affects its swimming performance, reproduction, mate selection, and foraging ability (Cooper et al., 2010; Head et al., 2013; Rowiński et al., 2015; Walker, 2010). Some populations of fish inhabiting warmed habitats in the wild have been found to show morphological divergence from ambient temperature populations (Lema et al., 2019; Pilakouta et al., 2023; Rocamontes-Morales et al., 2021; Rowiński et al., 2015). This divergence typically involves a deepening of the body, potentially indicating a shift to a more benthic lifestyle (Willacker et al., 2010). This result may relate to shifts in prey selection or availability in warmed habitats. Brown trout (*Salmo trutta*) inhabiting geothermally warmed streams become more selective in prey choice at higher temperatures, shifting towards more nutrient rich prey (O'Gorman et al., 2016). This may represent a behavioural adaptation to the increased energy demands of a warmed environment, which then leads to morphological change.

1.3 The three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus*) is a species complex that comprises populations of temperate freshwater or anadromous fish found throughout the northern hemisphere (Mckinnon and Rundle, 2002) Three-spined stickleback (hereafter stickleback) are incredibly ecologically diverse,

and are found in a wide variety of habitats including coastal, brackish, lake and river waters (Mckinnon and Rundle, 2002). This fish has been of particular note to evolutionary biologists due to the great morphological variation and ecological versatility it displays across its range (Baker et al., 2005; Wund et al., 2008). As a highly-evolvable species the stickleback provides an ideal candidate for investigating the potential routes of adaptation to climate-change driven temperature increases. This is particularly true of populations of stickleback found in Iceland. Geothermal activity in Iceland has created a large number of geothermally warmed freshwater habitats that can often be found within very close proximity to ambient temperature habitats. Several of these geothermal-ambient habitat pairs have been found to be inhabited by sticklebacks (Millet et al., 2013; Pilakouta et al., 2020, 2023; Strickland et al., 2023), presenting a unique opportunity to study thermal adaptation in the wild. Geothermal habitats are particularly advantageous for the research of thermal adaptation, providing a strong thermal gradient within a small spatial distance, there-by limiting the confounding factors that affect studies of thermal adaptation across latitudes and altitudes (e.g., photoperiod, seasonality, genetic drift and geology). Sticklebacks inhabiting geothermal waters in Iceland have been found to be consistently divergent from ambient sticklebacks in a range of traits, including morphology (Pilakouta et al., 2020, 2023) and metabolic rate.

1.4 Thesis Aims and Overview

In this project I aimed to investigate evolution and plasticity in three-spined sticklebacks putatively adapted to different thermal habitats in Iceland, using complementary field and laboratory-based experiments.

Chapter 2: Experimental evidence for adaptive divergence in response to a warmed habitat reveals roles for morphology, allometry, and parasite resistance.

While geothermal sticklebacks have been found to be consistently and heritably divergent in morphology, no direct test of the effects of this divergence on fitness have been performed. In this experiment, I first used a reciprocal transplant experiment to directly test for evidence of adaptive divergence between geothermal and ambient sticklebacks. I then tested for a relationship between morphology and fitness-proxies in the reciprocal

transplant, aiming to provide an insight into whether geothermal and ambient morphology provides an advantage in their respective habitats.

Chapter 3: Divergent gene expression and thermal plasticity in geothermal, ambient and hybrid stickleback

In this experiment I used whole transcriptome gene expression analysis to assess the gene mechanics behind divergence in gene expression and plastic responses between geothermal and ambient sticklebacks in an allopatric population pair.

Chapter 4: The consistency of divergence and plasticity of gene expression across multiple populations of geothermal sticklebacks

This experiment investigated the divergence in gene expression between geothermal and ambient sticklebacks across multiple populations. I tested for evidence of consistent, and therefore potentially adaptive, divergence between geothermal and ambient sticklebacks. I also tested for adaptive divergence in plastic responses in gene expression between geothermal and ambient sticklebacks.

2 Chapter 2: Experimental evidence for adaptive divergence in response to a warmed habitat reveals roles for morphology, allometry, and parasite resistance.

2.1 Introduction

The effects of climate change are expected to worsen within the next century, with a minimum of 1.5°C rise in the average global mean surface temperature (Lee et al., 2021). It is likely that increases in temperature will impose strong selection pressures due to effects on fundamental biological processes. Species tend to adapt to specific temperature ranges, with an organism's ability to function dropping off at extremes (Pörtner and Farrell, 2008). As most fish are ectotherms, they are vulnerable to temperature changes, and increased temperatures could pose a danger to their survival (Becker et al., 2018; Dudgeon et al., 2006; Pörtner and Knust, 2007). Even seemingly small increases in temperature that still allow for survival and reproduction could affect the performance of individuals and therefore lead to reduced fitness of individuals within fish populations, as constraints and trade-offs are placed on other traits. For example, temperature is an important factor for the growth of young fish (Friedland et al., 2005; Hurst et al., 2012; Teal et al., 2008; Todd et al., 2008), the survival of juveniles (Beaugrand et al., 2003) and this can trade-off with their size and age at maturity (Attrill and Power, 2002; Cox and Hinch, 1997; Jonsson and Jonsson, 2004; Otero et al., 2012; Ottersen et al., 2006; Rogers et al., 2011; Schindler et al., 2005).

Temperature changes are also likely to affect host-parasite interactions which will impact population fitness. For example, *Schistocephalus solidus* is a cestode macroparasite that infects stickleback as its second intermediate host (Hopkins and Smyth, 1951). Proportionate to body size, *S. solidus* infection can be extreme with parasite mass reaching up to 92% of that of its host (Hopkins and Smyth, 1951). This comes at great cost to the host, including reduced growth, reproductive capability, impaired immune system function and increased risk of predation via behavioural manipulation (Barber et al., 2004; Franke et al., 2019; Grécias et al., 2018; Heins and Baker, 2003). The optimal temperature for *S. solidus* is higher than that of the host (Franke et al., 2017,

2019; Macnab and Barber, 2012) and there is evidence that the parasite can alter the thermal preferences of stickleback, where they seek out warmer waters (Macnab and Barber, 2012). Increased environmental temperatures may therefore benefit the parasite while negatively affecting the fitness of the host. Exactly how a mismatch in thermal optima between parasite and host could be compounded with the additional pressures from climate change is an open question.

Furthermore, increased environmental temperature not only impacts fish directly, but can also drive indirect effects by altering ecosystems. Indeed, geothermal warmed and ambient temperature freshwater habitats differ in food web size, prey type and availability, structure and complexity (O'Gorman et al., 2012). Prey selection in fish is also impacted by temperature, potentially altering food web dynamics (O'Gorman et al., 2016). Such thermally-induced changes in prey availability and optimal feeding strategy could interact to influence selective pressures under a novel thermal habitat, particularly on morphological traits that are known to influence foraging success and swimming ability. These impacts are especially difficult to account for in lab experiments which only alter temperature.

2.1.1 Impact of thermal conditions on phenotypes

While physiological and life history traits have been a focus for researchers interested in the adaptation of fish to novel thermal habitats (Crozier and Hutchings, 2014), temperature changes are likely to cause broad phenotypic effects. For example, adaptive morphological variation has been intensively studied in fish in other contexts, where it has been shown to relate to swimming performance, reproduction, mate selection, and foraging ability (Cooper et al., 2010; Head et al., 2013; Rowiński et al., 2015; Walker, 2010). Wild populations living in natural or man-made warmed habitats show morphological divergence when compared to non-warmed temperature populations (Lema et al., 2019; Pilakouta et al., 2023; Rocamontes-Morales et al., 2021; Rowiński et al., 2015). Growing evidence from lab experiments shows that fish morphology can be phenotypically plastic in response to temperature (Corral and Aguirre, 2019; Georga and Koumoundouros, 2010; Georgakopoulou et al., 2007; Marcil et al., 2006; Ramler et al., 2014; Sfakianakis et al., 2011), while evidence of heritable divergence in morphology related to temperature

gradients have also been found (Marcil et al. 2006; Pilakouta et al. 2023). Most commonly seen in both lab and field examples is a deepening of body shape with increased temperature (Georgakopoulou et al., 2007; Lema et al., 2019; Marcil et al., 2006; Pilakouta et al., 2023; Rowiński et al., 2015). This consistency in morphological divergence with temperature suggests that such changes could be adaptive (Pilakouta et al., 2023), and/or influenced by biased developmental responses (Parsons et al., 2020). Temperature related morphological variation is relatively new, and tests of its fitness consequences, such as those performed by Ackerly and Ward, (2016), have been rare.

Furthermore, the assessment of the relationship between morphological variation and thermal habitat could benefit from a developmental perspective to inform evolutionary outcomes (Campbell et al., 2017, 2021). Indeed, temperature commonly affects growth rates in fish (Brett, 1979), suggesting that scaling relationships (allometry), which arise from differing relative growth rates between body parts, or between morphological traits and size, could be impacted (Casasa and Moczek, 2019; Savageau, 1979). Allometry itself is known to evolve in response to selection (Houle et al., 2019; Pélabon et al., 2014), suggesting it could be influenced by novel thermal habitats. Temperature-induce body shape variation in fish can be dependent on size (Lema et al., 2019; Rowiński et al., 2015). However, whether such allometry adaptively diverges in response to thermal conditions is unclear.

2.1.2 Wild systems for studying thermal effects on phenotypic variation.

Slow or inadequate adaptation to even low-level warming could result in an increased vulnerability to a range of threats (Becker et al., 2018), and possibly local extinction. While populations may simply leave unfavourable conditions in some cases, some populations face particular risk, such as in freshwater fishes where there can be relatively few opportunities to migrate away (Dudgeon et al., 2006). Therefore, it will be important to test how fish populations in nature, where both direct and indirect effects of warming exist, currently adapt. While rare, some systems can enable us to examine changes within predicted future conditions. For example, geothermal habitats offer a unique opportunity for studying the effect of temperature on a range of extant organisms (O'Gorman et al., 2014; Pilakouta et al., 2020). The wide-ranging thermal gradients that can occur over short physical distances within these

habitats allow for comparisons of 'geothermal' and 'ambient' populations without the same degree of confounding factors (e.g., photoperiod, geology, overall ecosystem makeup, and large amounts of genetic drift between populations) found over large latitudinal or altitudinal gradients. Given that climate change will affect whole habitats within a place, such study systems are a highly valuable complement to laboratory-based experiments.

Geothermal habitats are relatively common in Iceland, resulting in extreme temperature gradients, sometimes across just a few meters and often with no physical barriers between them (Jónasson et al., 1977; O'Gorman et al., 2012, 2014). Prior research has identified several of these habitats populated by the threespine stickleback (*Gasterosteus aculeatus*) (Pilakouta et al., 2020) – a well-known model species for ecology and evolution (Hendry et al., 2013). In these locations ambient and geothermal habitats can differ by more than 10^{o}C at a given time (Millet et al., 2013; Pilakouta et al., 2020). Ongoing research has found that these geothermal and ambient habitat sourced stickleback populations differ in diet, sociability, morphology and metabolic rate (unpublished data, Pilakouta et al., 2020, 2022, 2023). Heritable morphological divergence between these stickleback takes the form of a deeper body, more subterminal mouth, steeper craniofacial profile and a longer second dorsal spine in geothermal sourced fish (Pilakouta et al., 2023). Furthermore, the repeated nature of this divergence across independent populations suggests that adaptative divergence has occurred, but no direct experimental tests of adaptation have been performed.

Here, using stickleback populations inhabiting geothermal and ambient thermal habitats, I tested for evidence of adaptive divergence. Specifically, using a field-based reciprocal transplant experiment (Blanquart et al., 2013; De Villemereuil et al., 2016). I predicted 1) that indicators of fitness, would be suggest improved fitness, in the form of higher growth and survival, and lower parasite prevalence, when fish were within their native habitat relative to a non-native habitat. Parasite prevalence may also have a more complex interaction, due to the likelihood that the conditions of the geothermal habitat will benefit parasite fitness over that of the host fish, and the potential for geothermal sourced fish to have adapted to this pressure. I predict 2) that morphological differences would relate to growth. I also predicted 3) that

increased growth in the non-native habitat would be associated with body shapes more similar to that of natively sourced fish. Finally, I predicted 4) that morphological allometry could have an impact on growth.

2.2 Methods

2.2.1 Study system

I tested for adaptive thermal habitat divergence by performing a reciprocal transplant experiment at Áshildarholtsvatn (65°43'30.3"N, 19°36'02.5"W, Figure 2-1a) – a lake located near Sauðárkrókur in Northern Iceland. Adjoining Áshildarholtsvatn (ASHN) is a small pond fed by hot-water runoff (source temperature ~45.5°C) from nearby residential geothermal heating, houses built in the 1940s, installed within the past 70 years (Figure 2-1b). This pond eventually flows into a stream that runs off from the lake, creating an abrupt thermal gradient between the geothermal and ambient habitats (Figure 2-1c and d). The pond created by the hot water runoff (hereafter called the geothermal habitat) experiences temperatures around 10°C degrees higher than the ambient-water stream and lake (hereafter called the ambient habitat) year-round (Pilakouta et al., 2020). At the start of the experiment in June 2019 the temperatures were an average of 15.8ºC for the geothermal habitat and 8.7ºC for the ambient habitat.

The ambient habitat is larger and deeper than the geothermal habitat and differs in water chemistry in the concentrations of phosphate, magnesium, iron, silica and calcium, but is otherwise similar in measured parameters (Table 2-1). Despite the relatively young age of this system, and the lack of a physical barrier between the two habitats, evidence of heritable morphological divergence has been found between geothermal and ambient habitat stickleback (Pilakouta et al., 2023).

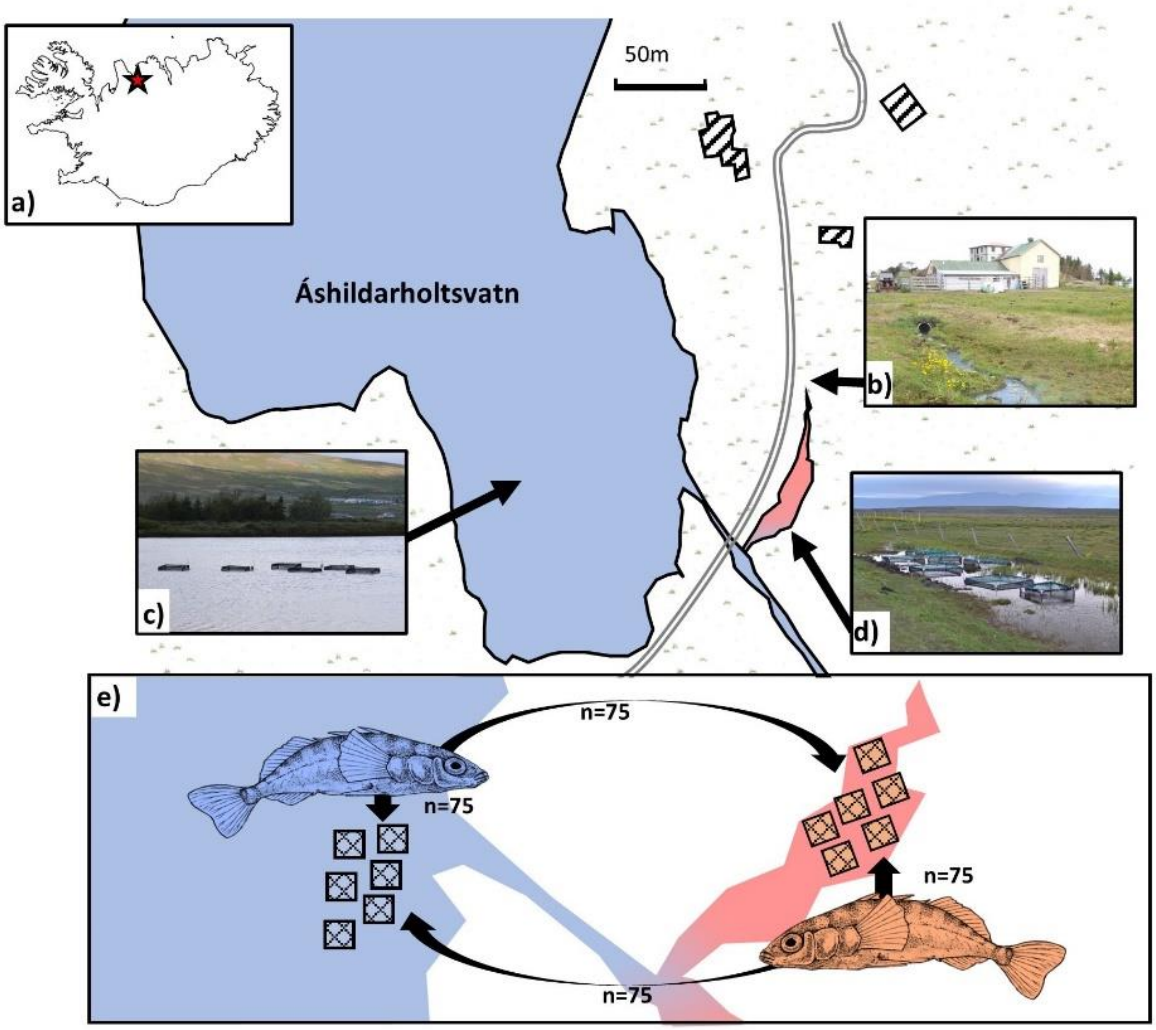


Figure 2-1 Geothermal-ambient study system used in this experiment a) Location of the Áshildarholtsvatn habitat pair in the North of Iceland (red star), b) Photograph showing the warm water outlet pouring into the warm habitat, c) cold habitat with cages, d) warm habitat with cages, e) experimental design of the reciprocal transplant experiment, showing transplantation of warm (pink) and cold (blue) sticklebacks to their native and opposing habitats.

Table 2-1 Water chemistry and seasonal temperatures of the warm and cold thermal habitats at Áshildarholtsvatn (measured 2016/17) and temperatures measured during the experiment (21/06/2019 – 18/07/2019)

													TEMP DURING EXPERIMENT		
SITE	**Coordinates**	**Summer temp (ºC)**	**Winter temp (ºC)**	**Dissolved Oxygen (mg/l)**	**TDS (ppm)**	**Sulfide (mg/L)**	**Phosphate (mg/L)**	**Magnesium (mg/L)**	**Iron (mg/L)**	**Silica (mg/L)**	**Hardness Ca2+ (mg/L)**	**Hardness (Trans %)**	**Mean (stdev) (ºC)**	**Max (ºC)**	**Min (ºC)**
ASHN WARM	65.72516 -, 19.600725	24.1	12.5	13.4	128	<0.1	0.1	<0.5	0.345	50.5	<3.0	92.0	21.76 (3.09)	27.5	14.0
ASHN COLD	65.724973 -, 19.602784	12.2	3.4	12.2	97	<0.1	0.02	>10.0	0.118	2.7	22.8	60.7	14.79 (2.17)	18.7	10.7

2.2.2 Fish collection and processing.

A total of 430 fish from each habitat were caught over three days using un-baited minnow traps, laid for 24 hours. The geothermal and ambient source fish were housed separately in four 20L buckets at Verið, Hólar University Research Station, in a flow through system under a temperature of 12.5°C (±1°C) which was intermediate to field site conditions. Fish were fed with 0.4 mm aquaculture feed every other day. Fish with noticeable swelling due to *Schistocephalus solidus* infection were excluded, although it was not possible to accurately determine infection status prior to the experiment. To increase the scope for growth during the experiment, I chose juvenile sticklebacks of a specific size range and lacking signs of sexual maturity (gravidity or male colours). Populations of wild stickleback vary considerably in standard length at sexual maturity, with reported size ranges typically contained within 32mm and 90mm (Baker et al., 2015; DeFaveri and Merilä, 2013; Millet et al., 2013; Narver, 1969; Walker, 1997). Mature stickleback captured from another Icelandic lake have been found to average 53.6 ± 1.01mm from a geothermal habitat, with stickleback from their corresponding ambient habitat averaging 55.0 ± 1.01mm (Millet et al., 2013). From lab-reared F1 Áshildarholtsvatn stickleback I observed average lengths of 53.9 mm (stdev 6.74) and 58.8mm (stdev 4.75) in sexually mature ambient and geothermal sourced stickleback respectively. Therefore, I chose a mean starting length of 40.6mm for fish sourced from both habitats(stdev 3.4), which corresponded with an average weight of 0.75g (stdev 0.22).

Within three days of capture the experimental fish were anaesthetised using phenoxyethanol, weighed and photographed (for morphometric analysis and standard-length measures) using a Nikon D3100 camera (Nikon, Tokyo, Japan) and then tagged with two visual implant elastomer tags (Northwest Marine Technology, Inc., Anacortes, USA) for re-identification following the conclusion of the reciprocal transplant experiment. The tagged fish were allowed to recover in captivity (minimum 10 days, maximum 13 days), with any dead fish being removed, identified, and replaced with a newly tagged fish if necessary (36 fish were replaced in this manner). Fish were not re-weighed after their time in the lab in order to reduce handling time and stress as much as possible.

2.2.3 Cage set up.

Reciprocal transplant cages consisted of black 5mm Fryma Mesh (Collins nets LTD, Dorset, UK) stretched over a cuboid skeleton of 32mm PVC pipes. The 5mm hole size of the Fryma Mesh was selected to allow for small fish to be used in the experiment, while also allowing invertebrates to pass through, as in previous stickleback transplant experiments (Hatfield and Schluter, 2006; Kaufmann et al., 2017; Stutz et al., 2015). The six cages intended for the ambient habitat were 1m x 1m x 1m, while the six geothermal habitat cages were of approximately the same volume but were 1.42m x1.42m x 0.5m in dimensions. These differences in dimensions were due to the shallow nature of the geothermal habitat. Geothermal habitat cages were placed close to the inlet of geothermal water where sticklebacks were naturally found and spaced approximately 50cm apart to allow for water flow. Ambient habitat cages were placed ~1m apart at the shore of the lake at a depth just short of 1m. All cages were seeded with sediment from their habitats and one cluster of native plants to provide shelter. All cages were then left for four days after placement to allow sediment to settle until the water was clear.

2.2.4 Reciprocal transplant

Geothermal and ambient sourced fish were alternated across a set of cages in both habitats to evenly distribute treatment types across possible environmental gradients. Three replicate cages were used for each of the four treatment types (ambient source fish transplanted to the geothermal habitat, geothermal to ambient, geothermal to geothermal, and ambient to ambient). Tagged fish were selected haphazardly from those housed in the lab but tag codes for each individual in a cage were recorded to enable re-identification. Each cage housed 25 fish, resulting in 75 fish per treatment type and a total of 300 fish used in the experiment (Figure 2-1e). Selected fish were released into the cages which were then covered with Nylon anti-bird netting with 22.5mm^2 holes. The transplant experiment ran for 30 days from mid-June to mid-July of 2019, with a take-down period of five days. During the experiment, all cages were checked three to four times a week (via visual inspection from the shore to reduce disturbance) for structural integrity and the habitat temperature measured using a digital thermometer at three points along the shore of each habitat. At the end of the experiment, stickleback were collected from cages

with the use of un-baited minnow traps checked hourly. The cages were then removed from the habitat, and the sediment within the cages checked for the presence of remaining fish. All cages were found to be intact at the end of the experiment; thus, unrecovered fish were assumed to have died. Recovered fish were transported back to the laboratory, euthanised with an overdose of phenoxyethanol, re-identified by elastomer tags, re-photographed for length measurements, and weighed. Fish were then dissected for assessment of *Schistocephalus solidus* parasite status (infected/uninfected). As this was only a preliminary investigation no further parasite data was recorded.

2.2.5 Gathering morphological data

To assess morphological variation, landmarks were collected for each fish from photographs taken prior to the start of the experiment using TPSdig2 version 2.31 (Rohlf, 2008). To minimize potential biases in landmarking, photographs were randomly arranged using the *randomly order specimens* function in TPSUtil 1.78 (Rohlf, 2015). A total of 27 landmarks (LMs) and a curve of 15 sliding semi landmarks were placed on each fish (Figure 2-2) with an eye to capture shape variation found to be important in geothermal-ambient stickleback divergence (Pilakouta et al., 2023). In a small number of photos (n=11) the mouth of the fish was slightly open affecting one landmark (landmark 1, Figure 2-2). Also, one ambient to geothermal transplant fish was found to be missing the first dorsal spine (landmark 6). Therefore, in these cases the affected landmark was designated as missing and its position estimated using the thin-plate spline method to estimate missing landmarks following Adams et al. (2020) using the *missing.landmark* function in the R package *Geomorph* version 3.1,3 (Adams and Otárola-Castillo, 2013; Adams et al., 2020). A small number of fish had more than three dorsal spines, in which case only the first three spines were landmarked. Body length was measured as standard length using the distance between landmarks 2 and 10 (Figure 2-2).

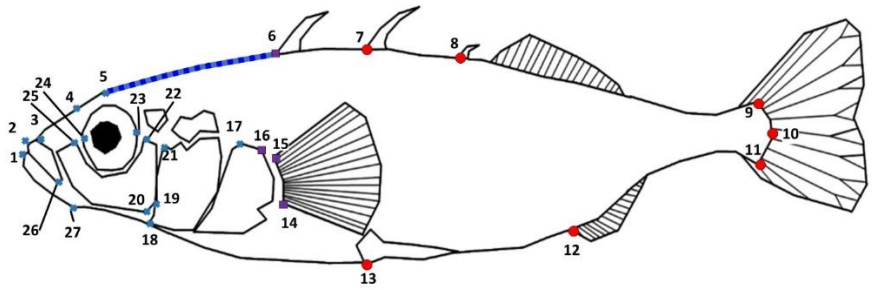


Figure 2-2 The 27 landmarks and semilandmark curve used in analysis of stickleback shape. 1 – anterior tip of the mandible, 2 – anterior tip of premaxilla, 3 – maxilla, 4 – nares, 5 – frontal, directly above eye, 6, 7 & 8 – anterior bases of first, second and third dorsal spines, 9, 10 & 11 – caudal peduncle, 12 – anterior base of anal spine, 13 – base of pelvic spine, 14 & 15- insertion points of pectoral fin, 16 & 17 – dorsal and anterior corners of pectoral girdle, 18 – junction of head to body on ventral midline, 19 & 21 – ventral and dorsal anterior corners of operculum, 20 & 22- ventral and dorsal corners of pre-operculum, 23 & 24 – posterior and anterior edge of eye, 25 – ventral corner of lacrimal, 26- Posterior end of premaxilla, 27 – posterior end of angular. 15 sliding landmarks were placed between landmarks 5 and 6 (blue line) for forehead morphology. Blue crosses are head landmarks, red circles are body landmarks, purple squares were used in both head and body data set.

2.2.6 Data analysis

2.2.6.1 Testing for evidence of adaptive divergence

All statistical analyses were conducted within the R 4.2.2 statistical language (R Core Team, 2022). I report findings with an alpha value between 0.1 and 0.05 as suggestive, but 0.05 was used as an indicator of statistical significance for all analyses. To test whether fitness proxies would improve when fish were within their native habitat (prediction 1), I used survival, parasite infection, and growth as response variables. Survival and parasite presence were measured as binary variables and chi-squared tests were used to test for differences between treatment groups. Further analysis using binomial family GLMs tested for the effects of transplant treatment on each of survival and parasite infection. A simple candidate model was created for each analysis, with the variables of source habitat, destination habitat and the interaction between the two, as shown below.

$$survival \sim source\ habitat * destination\ habitat$$

$$parasite\ infection\ status \sim source\ habitat * destination\ habitat$$

A further fourteen candidate models were created for each analysis, based on the simple model, each including and excluding additional variables to control for the effects of starting size (starting weight and length), and cage effects (cage nested within destination) and potential interactions between each (supplementary table 1). Model selection was performed and based on AIC values obtained from the *AIC* function in R (package *stats* version 4.2.2 (R Core Team, 2022)). Models with the lowest AIC value were taken to represent the best fit to the data, but when two models had AIC value within two of each other I selected the simpler model following Burnham and Anderson (2002). Model permutations and selected models are shown in supplementary table 1.

Growth, measured as changes in weight and length, was also examined in relation to the impacts of source, destination, and their interaction using GLMs. However, to account for the possibility that fish of different starting sizes would exhibit varying growth rates I first standardized changes in weight and length against their respective size at the beginning of the experiment using a linear regression to obtain residuals that were then used for GLMs. Linearity of growth was checked by examining fitted vs residual plots and was found to be linear, therefore no log transformation was applied. A simple candidate model was created for each analysis, with the variables of source habitat, destination habitat and the interaction between the two, as shown below.

$$residual\ weight\ change \sim source\ habitat * destination\ habitat$$

$$residual\ length\ change \sim source\ habitat * destination\ habitat$$

A further five permutations of each of these models including and excluding additional variables to control for cage effects (cage nested within destination) and parasite effects (*S. solidus* infection status) were created as a basis for model selection, which was performed in the same manner as for the binomial models (supplementary table 1).

Notably, weight and length change data were not combined into a single condition factor as this may reduce my ability to explain changes in size and could cause difficulties comparing populations with potentially different weight-length relationships (Froese, 2006). Additionally, because fish did not have *S. solidus* parasites removed before weighing, and due to the potentially substantial sizes of these parasites, weight values at the end of the experiment

(unlike standard length) may not have been wholly attributable to the weight of the fish alone. For both the binomial and GLM models, findings of interactions between source and destination effects would be indicative of adaptive fitness differences between geothermal and ambient sourced fish, supporting my prediction of adaptive divergence.

2.2.6.2 Testing relationships between growth and shape

As anatomical regions can have both differing functions and developmental origins (Parsons et al., 2011), and because body shape was potentially susceptible to influences from parasite prevalence, shape analysis was performed on three sets of landmark data: the head, the posterior body, and both combined (Figure 2-2, see also Wund et al., 2008). The landmarks used for the head subset were 1 – 6, 14 – 27, and the sliding landmark curve, while the posterior body subset consisted of landmarks 7 – 16, with both subsets comprising the whole fish, i.e., total body shape (Figure 2-2). Each set of landmarks was standardized for variation in size, translated and rotated to minimize interindividual landmark distances using a generalized Procrustes analysis implemented from the *gpagen* function in *Geomorph* with sliding semilandmark positions optimised to minimise bending energy.

To address prediction 2 and determine the relationship between morphology, my experimental factors and growth, each landmark set was modelled using a Procrustes ANOVA in *Geomorph*. Here, landmarks were used exclusively from fish that survived the experiment (n=269). For each Procrustes ANOVA source, destination, log centroid size (to account for static allometry), and one growth measure for each model (either residual weight or length change) were tested for effects on morphological shape using the *ProcD.lm* function with type III sums of squares (see base model design below).

$$Shape \sim residual\ weight\ change * source * destination * \log(centroid\ size)$$

$$Shape \sim residual\ length\ change * source * destination * \log(centroid\ size)$$

To allow for model selection and to assess whether there was a need to control for cage effect a second version of each model was also made with the additional variable of cage (nested within destination. Model selection was performed using a nested ANOVA through the *ANOVA* function (package *stats*

version 4.2.2) whereby if cage effects did not significantly change results the simpler model was chosen.

To address prediction 3, that increased growth in the non-native habitat would correspond to morphological shape resembling the average native shape, I visualized both 1) the average divergence in shape between geothermal and ambient source stickleback, and 2) the relationships between shape and growth measures (weight change or length change) using deformation grids. Deformation grids were created using the *PlotReftoTarget* function in *Geomorph*, which generates thin-plate spline deformation grids plotting the difference in shape of a target specimen in relation to a reference specimen. Thus, a deformation grid representing how geothermal sourced fish differed from an ambient sourced fish was created by using the mean shape of ambient sourced fish as a reference against the mean shape of geothermal sourced fish as the target. Also, differences in shape between the best and worst performing fish (measured as length change or weight change) in each treatment group were estimated from growth and deformation grids created from growth values found to relate to shape in my Procrustes ANOVAs. I used the *Geomorph* function *shape.predictor* to estimate shape against minimum and maximum growth values. Deformation grids were created where one extreme of the scale would represent the shape of fish with the "best" growth (e.g., greatest increase in length), while the other end of the scale represented the shape of fish with the "worst" growth (e.g., lowest increase in length). To accentuate differences, shape deformation was magnified by 3x.

2.2.6.3 Testing relationships between growth and allometry

To address prediction 4, that adaptive morphological variation could be attributed to allometry, I assessed the contribution of allometry to shape divergence by testing whether size/shape relationships were different between geothermal and ambient source fish. This was done as a follow up to my Procrustes ANOVAs to further understand whether potential interactions between size and shape were due to differential allometry between fish from different thermal sources. Therefore, to address whether size simply differed between fish from different thermal sources I first tested whether geometric centroid size and starting length differed between geothermal and ambient populations using a t-test (*t.test* function, package *stats* version 4.2.2). I also

tested for a difference in the variation of centroid size and starting length between geothermal and ambient populations using a Levene's test using the *leveneTest* function from the *car* package in R (Fox and Weisberg, 2019; Levene, 1960). Following this, a test for allometric differences between fish from the two thermal habitats was performed for each set of landmarks using ANOVA to compare a unique allometric *ProcD.lm* model (with an interaction between log centroid size and source) to a null common allometric *ProcD.lm* model (with no interaction between log centroid size and source). Due to the potential for *S. solidus* to alter the centroid size of fish, a similar model was created but used the starting length of fish as a measure of size. I also visualised allometric relationships using *Geomorph's plotAllometry* function to create two plots for each analysis. The first plot, using the option "*PredLine*", plots the first principal component of predicted values versus size from the calculated fitted values from the selected *procD.lm* fit in the test of allometric relationships (unique vs common). The second plot, using the option "*RegScore*", plots standardized shape scores, calculated from the regression of shape on size, against size.

Finally, to test for evidence of adaptive allometric variation I compared shape between survivors and fatalities. Specifically, rather than using all fish (n=300) this involved separately testing for differences in morphological shape between survivors and fatalities in the treatment experiencing the lowest survival rate (one treatment group, total sample size n=75) using a Procrustes ANOVA with survival and size (centroid size, and length) as explanatory variables. Finally, to rule out the possibility that survivor was determined by size a standard t-test of centroid size comparing fish that survived or died was performed using the *t.test* function. I also tested for a difference in the variation of centroid size and starting length between stickleback that survived or died during the experiment using a Levene's test from the *leveneTest* function within the *car* package in R (Fox and Weisberg, 2019; Levene, 1960). I visualized allometric shape variation that differed between survivors and fish that died in this group using deformation grids generated by predicting shape against minimum and maximum log centroid size and plotted with *Geomorph's PlotReftoTarget* using the same method as used for the previous deformation grids. These deformation grids were magnified by 3x to enable clear visualisation

of allometric divergence. Plots of allometric relationships were created using p*lotAllometry* as before.

2.3 Results

2.3.1 Testing for evidence of adaptive divergence

Overall, survival was high (89.7% of fish survived), but survival rates differed among transplant treatment types ($X^2_{(1, N = 300)} = 26.298$, $p < 0.05$). Ambient sourced stickleback transplanted to the geothermal habitat experienced the lowest survival rate at 74.7% as compared to ≥92% survival rates in other groups (geothermal to geothermal: 93.3%, ambient to ambient: 92% and geothermal to ambient: 98.7%; Figure 2-3a). The best supported survival binomial model showed that destination and source separately had an effect on survival (Table 2-2).

The prevalence of *S. solidus* differed between fish source and destination ($X^2_{(1, N = 269)} = 50.217$, $p < 0.05$). Fish caged in the geothermal habitat were more likely to be infected than fish caged in the ambient habitat with 40% of geothermal and 62.5% of ambient source surviving stickleback infected, while in the ambient habitat only 11.6% of ambient and 14.9% of geothermal source surviving stickleback were infected (Figure 2-3b). The selected binomial parasite prevalence model indicated effects from source, destination, and the interaction between source and destination, suggesting adaptive divergence in relation to parasite resistance (Table 2-2).

The transplant experiment also impacted fitness proxies, as measured through residual weight change (Table 2-3 and Figure 2-3c and d). Model selection favoured a GLM with source, destination, cage (nested within destination) and parasite infection status as factors, with adaptive divergence indicated through an interaction between source and destination (Table 2-3). Fish transplanted to the geothermal habitat were more likely to gain weight (mean residual weight change = 0.04g), while in the ambient destination, weight loss was more likely (mean residual weight changes = -0.06g) (Figure 2-3d). However, this was not equal across sources with fitted weight change indicating that geothermal sourced fish transplanted to the ambient habitat were more likely to lose weight than geothermal sourced fish in the native habitat (Appendix 1: Supplementary Figure 1).

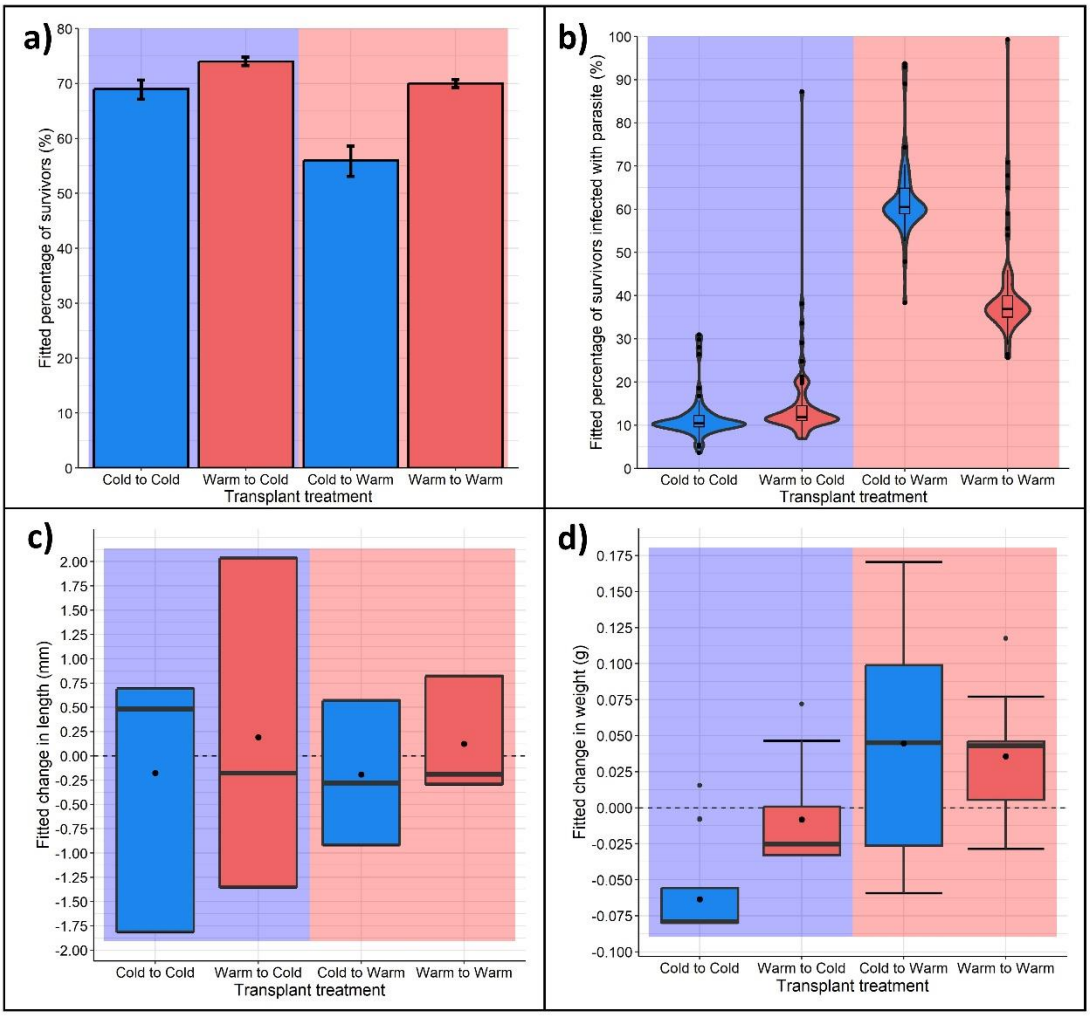


*Figure 2-3 . Fitted values of performance measures from model analysis for a) Survival (mean percentage of fish recovered per cage per treatment), where error bars represent the standard deviation between cages within treatments and b) Schistocephalus solidus prevalence (mean percentage of recovered fish found to be infected per cage per treatment). Treatment designated by the thermal source (warm and cold) of the fish and thermal habitat destination (warm and cold). As the simplest model design was selected for the survival analysis (survival ~ source*destination), no variation in fitted values was present between individuals within each treatment type. Box and whisker plots c) and d) display fitted variation in c) residual length changes (mm) and d) residual weight changes (g), that occurred with experimental treatments in the reciprocal transplant experiment involving natural cold and warm habitats. Top and bottom hinges represent 25th and 75th percentile, centreline represents 50th. Black dot displays mean residual weight change. Whiskers give 95% confidence interval. Blue and red filled boxes represent the cold and warm sourced fish respectively, blue and red backgrounds represent the cold and warm destination habitats, respectively.*

Table 2-2 Results of binomial family GLMs (model permutation selected by AIC) testing for the effect of a reciprocal transplant experiment on survival and parasite infection of threespine stickleback in Iceland. Rows highlighted in blue represent interaction combinations that address the question of whether the fitness proxy measure in question relates to transplant treatment.

BINARY FITNESS PROXY MEASURE AND VARIABLES

SURVIVAL	**Est.**	**Std. Error**	**Z**	**P**
Source	1.862	1.093	1.703	**0.089.**
Destination	-1.361	0.502	-2.714	**0.007****
Source x Destination	-0.304	1.216	-0.250	0.803
(*Intercept*)	2.442	0.426	5.738	**>0.001*****
PARASITE INFECTION	**Est.**	**Std. Error**	**Z**	**P**
Source	0.111	0.514	0.217	0.828
Destination	2.611	0.478	5.461	**<0.001*****
Source x Destination	-1.080	0.636	-1.698	**0.090.**
Start weight	-19.257	9.355	-2.058	**0.040***
Start length	-0.280	0.170	-1.644	0.100
Start weight x start length	0.495	0.230	2.152	**0.031***
(*Intercept*)	8.691	6.924	1.255	0.209

Table 2-3 Results of GLMs (permutation selected by AIC) testing the effect of the reciprocal transplant experiment of threespine stickleback in Iceland on growth measures (residual length and weight change). Rows highlighted in blue represent interaction combinations that address the question of whether the fitness proxy measure in question relates to transplant treatment.

FITNESS PROXY MEASURE AND VARIABLES					
RESIDUAL WEIGHT CHANGE		**Est. Coefficients**	**Std. Error**	**T value**	**P**
Source		0.054	0.027	1.975	**0.049***
Destination		0.178	0.031	5.710	**<0.001*****
Source x Destination		-0.181	0.041	-4.439	**<0.001*****
Parasite Infection Status		0.072	0.014	5.100	**<0.001*****
Cage (Nested within Destination)	*# 2*	-0.158	0.031	-5.024	**<0.001*****
	# 3	0.034	0.028	1.217	0.225
	# 4	-0.120	0.032	-3.728	**<0.001*****
	# 5	0.075	0.028	2.678	**0.008****
	# 10	0.026	0.027	0.958	0.339
	# 11	-0.001	0.028	-0.020	0.984
	# 12	-0.008	0.027	-0.284	0.7769
	# 13	0.023	0.028	0.825	0.4102
(Intercept)		-0.079	0.020	-4.049	**<0.001*****
RESIDUAL LENGTH CHANGE		**Est. Coefficients**	**Std. Error**	**T value**	**P**
Source		1.342	0.854	1.572	0.117
Destination		-0.969	0.947	-1.023	0.307
Source x Destination		-0.244	1.275	-0.191	0.849
Cage (Nested within Destination)	*# 2*	0.846	0.986	0.858	0.392
	# 3	-1.108	0.872	-1.270	0.205
	# 4	-0.661	1.105	-0.654	0.514
	# 5	-1.010	0.872	-1.158	0.248
	# 10	-2.209	0.845	-2.614	**0.009****
	# 11	-0.208	0.872	-0.239	0.811
	# 12	-3.381	0.854	-3.960	**<0.001*****
	# 13	-2.500	0.882	-2.834	**0.005****
(Intercept)		0.690	0.610	1.131	0.259

2.3.2 Testing relationships between growth and shape

The thermal habitat affected length change (i.e., growth) of geothermal and ambient source fish differently on the basis of head, posterior body and total body shape (indicated by a three-way interaction between source, destination, and the three subdivisions of shape (Table 2-4). Fish transplanted to the non-native habitat appeared to experience greater increases in length if their body shape resembled that of fish native to that habitat (

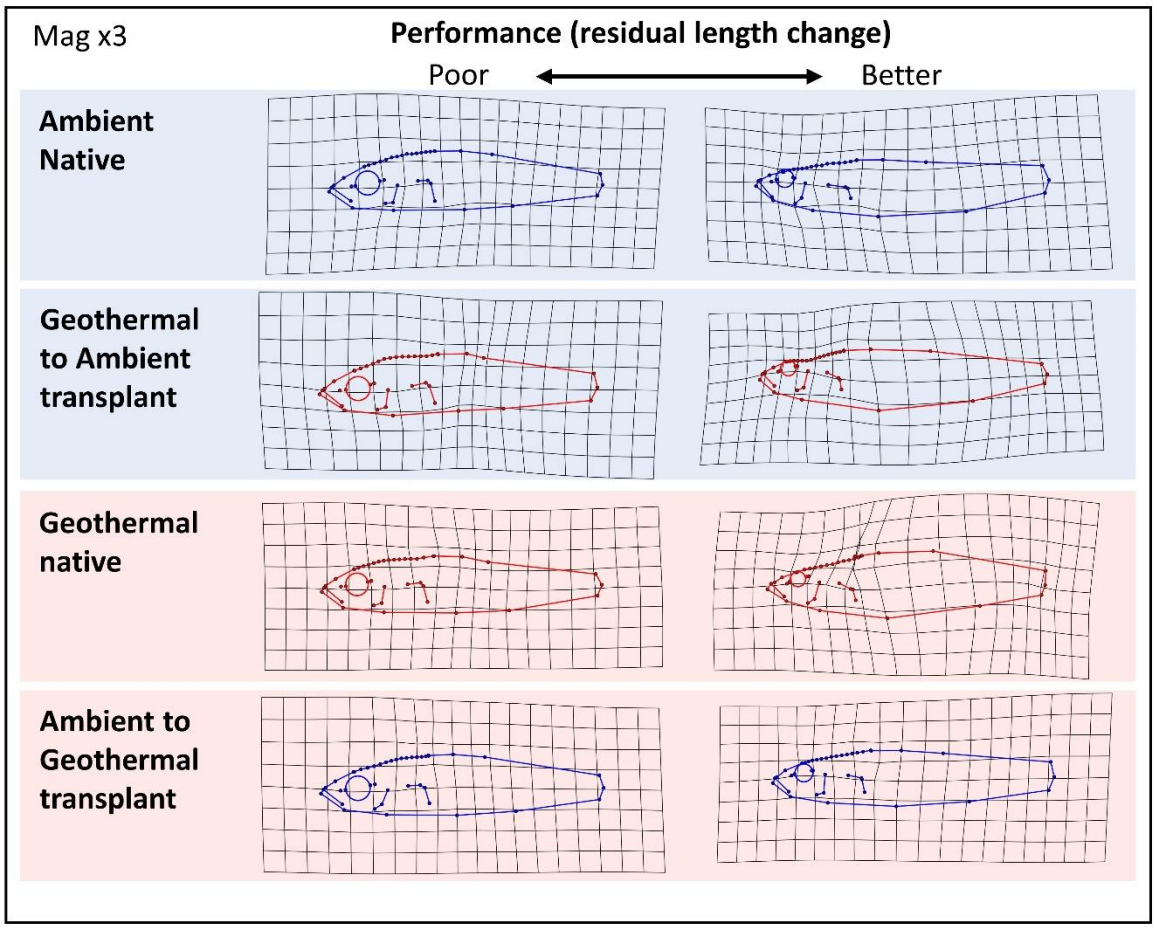


Figure 2-4). Geothermal sourced fish with the greatest length increases in the ambient habitat were those with narrower bodies, a more upward facing mouth

and a concave forehead shape (

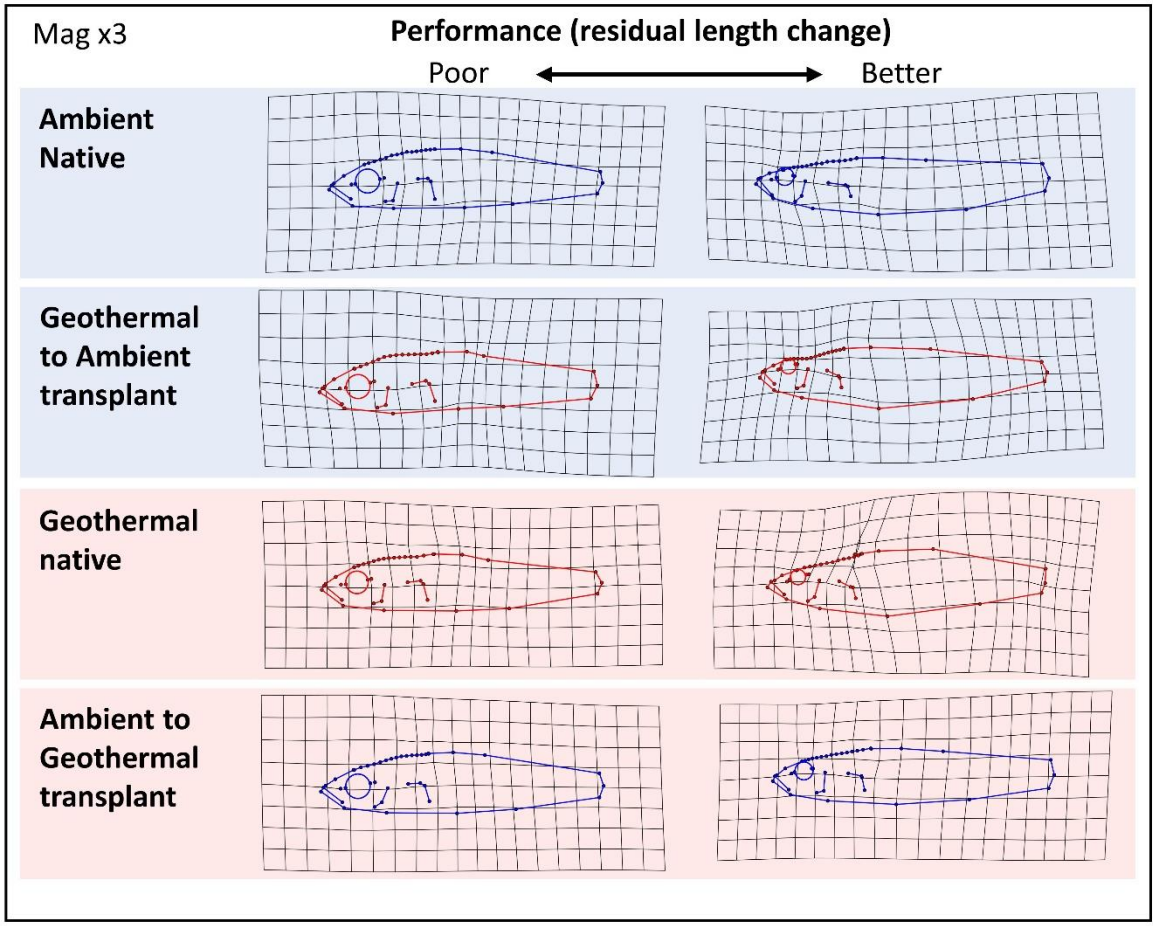


Figure 2-4). Ambient sourced fish transplanted to the geothermal habitat showed greater length increases with a slightly more subterminal mouth as compared to

ambient native fish (

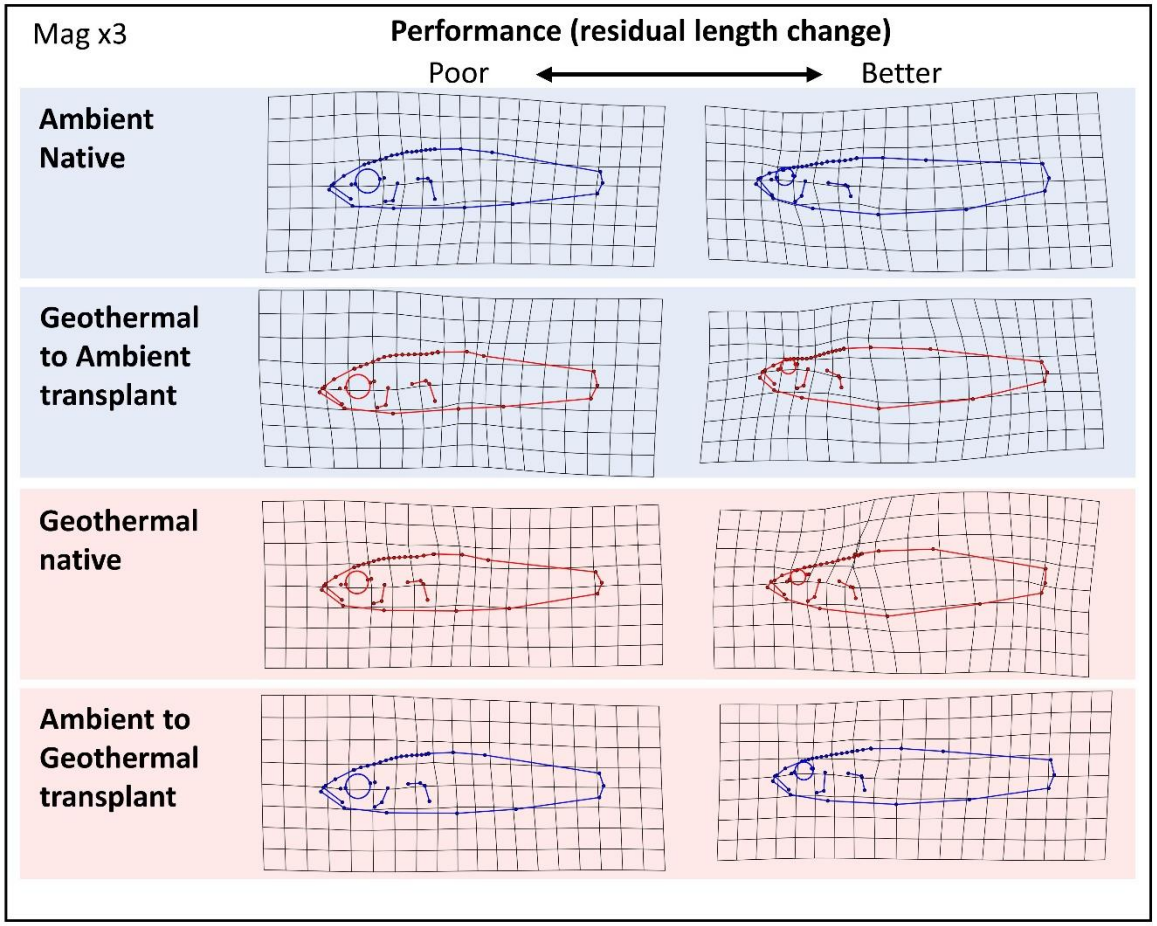


Figure 2-4).

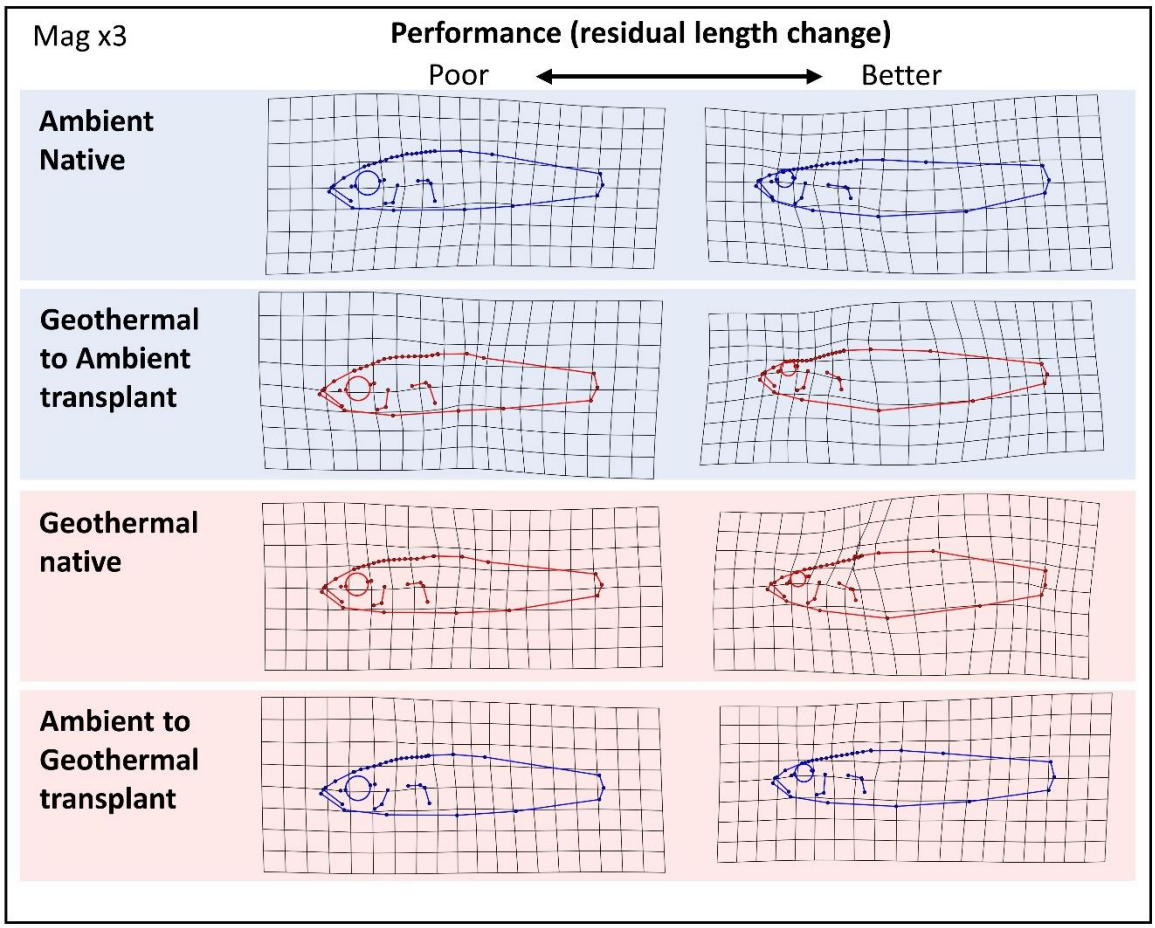


Figure 2-4 Deformation grids (with 3x magnification) visualising the worst (left) and best (right) performing fish, in terms of residual length change, across the transplant groups. Blue and red outlines represent cold and warm sourced sticklebacks respectively while blue and red background represent the cold and warm destination habitats, respectively.

Table 2-4 Results of Procrustes ANOVA shape analysis models with growth measures, for total fish, head, and body shape of threespine stickleback. Rows highlighted in blue represent interaction combinations that address the question of whether shape relates to growth and transplant treatment.

GROWTH MEASURE AND VARIABLES	TOTAL FISH SHAPE						HEAD SHAPE						POSTERIOR BODY SHAPE					
	df	F	Z	SS	PVE	P	df	F	Z	SS	PVE	P	df	F	Z	SS	PVE	P
Residual Weight Change (WC)	1	0.602	-0.621	0.0010	0.2	0.732	1	0.307	-1.264	0.0015	0.1	0.896	1	0.721	-0.338	0.0009	0.2	0.621
Source	1	1.024	0.352	0.0017	0.3	0.340	1	0.546	-0.461	0.0026	0.2	0.666	1	1.278	0.751	0.0016	0.4	0.220
Destination (Dest)	1	0.587	-0.687	0.0010	0.2	0.746	1	1.253	0.741	0.0060	0.4	0.245	1	0.647	-0.561	0.0008	0.2	0.709
Log centroid size (logCS)	1	2.738	2.032	0.0046	0.9	**0.022***	1	0.791	0.101	0.0038	0.3	0.464	1	4.251	3.343	0.0055	1.4	**0.001****
WC x Source	1	0.529	-0.753	0.0010	0.2	0.783	1	0.271	-1.556	0.0013	0.1	0.944	1	0.906	0.061	0.0012	0.3	0.469
WC x Dest	1	1.360	0.869	0.0023	0.5	0.183	1	1.201	0.694	0.0057	0.4	0.253	1	0.941	0.099	0.0012	0.3	0.462
Source x Dest	1	1.469	1.012	0.0024	0.5	0.170	1	2.201	1.394	0.0105	0.8	**0.088.**	1	0.383	-1.564	0.0005	0.1	0.935
WC x logCS	1	0.612	-0.587	0.0010	0.2	0.724	1	0.318	-1.208	0.0015	0.1	0.886	1	0.713	-0.359	0.0009	0.2	0.631
Source x logCS	1	1.017	0.340	0.0017	0.3	0.349	1	0.547	-0.461	0.0026	0.2	0.667	1	1.250	0.708	0.0016	0.4	0.231
Dest x logCS	1	0.612	-0.662	0.0010	0.2	0.736	1	1.223	0.714	0.0058	0.4	0.253	1	0.631	-0.611	0.0008	0.2	0.724
WC x Source x Dest	1	1.017	1.081	0.0025	0.5	0.142	1	1.349	0.812	0.0065	0.5	0.227	1	1.372	0.850	0.0018	0.4	0.206
WC x Source x logCS	1	0.595	-0.724	0.0010	0.2	0.776	1	0.284	-1.479	0.0014	0.1	0.929	1	0.891	0.031	0.0012	0.3	0.480
WC x Dest x logCS	1	1.390	0.895	0.0025	0.5	0.174	1	1.23	0.729	0.0059	0.4	0.246	1	0.930	0.077	0.0012	0.3	0.470
Source x Dest x logCS	1	1.482	1.028	0.0025	0.5	0.164	1	2.146	1.368	0.0103	0.8	**0.089.**	1	0.370	-1.628	0.0005	0.1	0.942
WC x Source x Dest x logCS	1	1.548	1.099	0.0026	0.5	0.140	1	1.365	0.828	0.0065	0.5	0.221	1	1.351	0.819	0.0017	0.4	0.215
Residuals	253			0.4200	85.0		253			1.2088	89.9		253			0.3257	82.5	
Residual Length Change (LC)	1	2.616	1.993	0.0043	0.9	**0.023***	1	0.793	0.170	0.0038	0.3	0.449	1	4.369	3.100	0.0052	1.3	**0.001****
Source	1	2.119	1.603	0.0035	0.7	**0.047***	1	0.657	-0.134	0.0031	0.2	0.546	1	4.201	3.030	0.0050	1.3	**0.002****
Destination (Dest)	1	1.883	1.355	0.0031	0.6	**0.093.**	1	0.339	-1.136	0.0016	0.1	0.875	1	4.601	3.506	0.0055	1.4	**0.001****
Log centroid size (logCS)	1	5.155	3.165	0.0084	1.7	**0.002****	1	1.128	0.626	0.0054	0.4	0.299	1	16.626	6.652	0.0199	5.01	**0.001****
LC x Source	1	2.164	1.661	0.0035	0.7	**0.052.**	1	1.045	0.546	0.0050	0.4	0.301	1	3.174	2.606	0.0038	1.0	**0.006****
LC x Dest	1	2.192	1.613	0.0036	0.7	**0.063.**	1	1.938	1.286	0.0092	0.7	0.100	1	3.021	2.425	0.0036	0.9	**0.007****
Source x Dest	1	1.192	0.581	0.0019	0.4	0.297	1	0.446	-0.699	0.0021	0.2	0.748	1	2.209	1.684	0.0025	0.6	**0.048***
LC x logCS	1	2.513	1.922	0.0041	0.8	**0.028***	1	0.851	0.272	0.0041	0.3	0.404	1	4.520	3.116	0.0054	1.4	**0.001****
Source x logCS	1	2.112	1.596	0.0034	0.7	**0.048***	1	0.674	-0.097	0.0032	0.2	0.528	1	4.145	2.998	0.0050	1.3	**0.002****
Dest x logCS	1	1.856	1.328	0.0030	0.6	**0.097.**	1	0.345	-1.111	0.0016	0.1	0.869	1	4.522	3.464	0.0054	1.4	**0.001****
LC x Source x Dest	1	2.394	1.731	0.0039	0.8	**0.048***	1	2.770	1.669	0.0132	1.0	**0.044***	1	3.176	2.629	0.0038	1.0	**0.005****
LC x Source x logCS	1	2.194	1.691	0.0036	0.7	**0.048***	1	1.130	0.647	0.0054	0.4	0.278	1	3.280	2.707	0.0039	1.0	**0.005****
LC x Dest x logCS	1	2.098	1.536	0.0034	0.7	**0.064.**	1	1.960	1.303	0.0093	0.7	0.101	1	3.026	2.411	0.0036	0.9	**0.008***
Source x Dest x logCS	1	1.176	0.558	0.0019	0.4	0.302	1	0.457	-0.665	0.0022	0.2	0.732	1	2.043	1.643	0.0025	0.6	**0.053.**

LC x Source x Dest x logCS	1	2.207	1.602	0.0036	0.7	**0.059.**	1	2.730	1.658	0.0130	1.0	**0.043***	1	2.943	2.457	0.0035	0.9	**0.008***
Residuals	253			0.4118	83.4		252			1.2035	89.5		253			0.3029	76.8	

2.3.3 Testing relationships between growth and allometry

Evidence of divergent shape allometry was detected in the total fish shape and body shape landmark sets (Table 2-5). In the case of body shape, the clearest difference in allometry between geothermal and ambient sourced fish was seen to be a greater increase in body depth with increasing size in geothermal sourced fish relative to ambient sourced fish (Supplementary Figure 2 a). Allometric divergence was further supported by the finding that size did not differ between geothermal and ambient source fish for both centroid size ($t_{(293)}$ = -1.476, p = 0.14) and fish length ($t_{(293)}$ = -1.135, p = 0.26). Geothermal and ambient source fish also did not differ in the amount of variation in centroid size ($F_{(298)}$ = 0.560, p = 0.455) or starting length ($F_{(298)}$ = 0.950, p = 0.331).

My data also indicated that survival was related to allometry. Indeed, the detection of divergent allometry was dependant on survival outcome (Table 2-5).For example, divergent head allometry was detected between geothermal and ambient source *surviving* fish, but not in the *full set* of experimental fish. This may indicate that fish with more intermediate size-shape relationships died during the experiment, suggesting that my experiment promoted divergent selection on head allometry. Deformation grids depicting allometry indicated that larger surviving geothermal sourced fish had a more subterminal craniofacial region relative to larger ambient sourced fish (Appendix 1: supplementary figure 2b). Differences in eye size between small and large fish also appeared to be more distinct in the surviving fish than in those that died. Size-shape relationships in total fish shape and in body shape show an opposite relationship, where unique allometry was detected in the full set of experimental fish, but not detected when fatalities were removed from the dataset.

Table 2-5 Unique vs Common allometry model ANOVA comparison tests for total fish, head and body shape of threespine stickleback in Iceland, using all fish and survivors only data sets. Common allometry model used as null; significant p value suggests unique allometry model is most appropriate.

	TOTAL FISH SHAPE					HEAD SHAPE					BODY SHAPE				
LOG CENTROID SIZE	**d**	**F**	**Z**	**SS**	**P**	**d**	**F**	**Z**	**SS**	**P**	**d**	**F**	**Z**	**SS**	**P**
All Fish	1	2.37	1.693	0.0042	**0.05**	1	1.10	0.551	0.005	0.302	1	2.51	2.049	0.003	**0.022***
Survivors Only	1	1.69	1.187	0.0029	0.121	1	2.96	1.624	0.014	**0.05**	1	1.62	1.148	0.002	0.112
LOG LENGTH AS SIZE															
All Fish	1	2.57	1.820	0.0045	**0.04**	1	1.36	0.787	0.007	0.236	1	3.64	2.708	0.004	**0.002***
Survivors Only	1	0.28	-	0.0005	0.984	1	0.42	-	0.002	0.779	1	0.24	-	0.000	0.990

Overall, as I reported above the rate of mortality was low during the experiment. This limited follow-up analysis further tested relationships between allometry and survival to the ambient source to geothermal destination transplant treatment group (lowest survival rate of 74.7%). Allometry was associated with survival in the ambient source to geothermal destination transplant group (Table 2-5). Here, Procrustes ANOVAs indicated that survival was related to total fish and body shape when interacting with size (for both log centroid size and log length). Among my factors this interaction explained the most variation in the total fish shape model (3.1-3.3% percentage variation explained (PVE)), and the second most in the body shape model (3.0% PVE) (Table 2-5). Survivors and fatalities did not differ in centroid size ($t_{(26)} = 0.255$, $p = 0.80$) or starting length ($t_{(26)} = 0.312$, $p = 0.76$) indicating that this finding was due to a relationship between size and shape. Also, variation in centroid size did not differ between survivors and fatalities ($F_{(73)} = 0.839$, $p = 0.363$), nor did starting length ($F_{(73)} = 1.017$, $p = 0.316$) indicating this finding was not driven by a lack of variation in one group.

Larger ambient to geothermal survivors possessed a greater body depth, shorter craniofacial region, and a smaller eye than larger fatalities, while small survivors had a more fusiform body profile and larger eye than smaller fatalities (Figure 2-5).

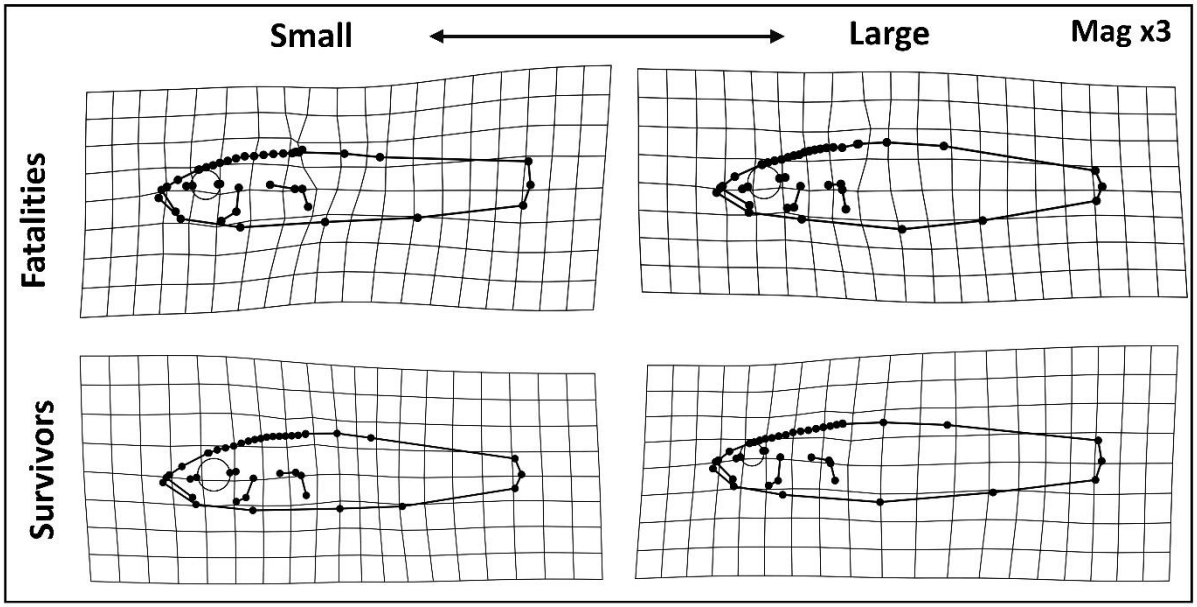


Figure 2-5 Deformation grids (with 3x magnification) depicting allometric relationships as shape extremes related to centroid size for the surviving and non-surviving cold source to warm habitat transplant fish.

Table 2-6 Results of Procrustes ANOVA shape analysis of the cold to geothermal transplant group, with survival and log centroid size as explanatory variables. P values below 0.1 highlighted in bold. Rows highlighted in blue represent interaction combinations that address the question of whether shape relates to survival and log centroid size.

	TOTAL FISH SHAPE						HEAD SHAPE						BODY SHAPE					
LOG CENTROID SIZE AS SIZE	**df**	**F**	**Z**	**SS**	**PVE**	**P**	**df**	**F**	**Z**	**SS**	**PVE**	**P**	**df**	**F**	**Z**	**SS**	**PVE**	**P**
Log CS	1	1.312	0.752	0.0028	1.7	0.244	1	3.539	1.844	0.0249	4.7	**0.027***	1	1.116	0.418	0.0011	1.4	0.331
Survival	1	2.331	1.642	0.0051	3.1	**0.058.**	1	2.376	1.434	0.0167	3.1	0.088.	1	2.504	2.021	0.0024	3.2	**0.021***
Log CS x Survival	1	2.332	1.641	0.0051	3.1	**0.056.**	1	2.335	1.415	0.0164	3.1	0.089.	1	2.541	2.053	0.0025	3.3	**0.021***
Residuals	71			0.1454	94.2		71			0.5000	93.5		71			0.0692	91.4	
LOG LENGTH AS SIZE	**df**	**F**	**Z**	**SS**	**PVE**	**P**	**df**	**F**	**Z**	**SS**	**PVE**	**P**	**df**	**F**	**Z**	**SS**	**PVE**	**P**
Log L	1	1.323	0.770	0.0029	1.8	0.236	1	1.925	1.184	0.0139	2.6	0.140	1	1.259	0.667	0.0013	1.7	0.256
Survival	1	2.424	1.710	0.0053	3.2	**0.048***	1	1.567	0.952	0.0113	2.1	0.198	1	2.292	1.815	0.0023	3.0	**0.033***
Log L x Survival	1	2.435	1.704	0.0053	3.2	**0.048***	1	1.541	0.934	0.0111	2.1	0.203	1	2.328	1.848	0.0023	3.1	**0.028***
Residuals	71			0.1545	94.4		71			0.5118	95.7		71			0.0708	93.4	

2.4 Discussion

I tested for fitness proxy consequences of divergence between geothermal and ambient sourced stickleback, and for an effect of divergent morphology on growth in a field reciprocal transplant experiment. I found evidence of negative consequences for fish transplanted to the non-native habitat, with further preliminary evidence suggesting that divergent shape and allometry may play a role in how well fish perform when transplanted.

2.4.1 Testing for evidence of adaptive divergence

My findings support my first prediction, that stickleback should perform best in their native habitat, suggesting that adaptive divergence has occurred (Blanquart et al., 2013; De Villemereuil et al., 2016). However, there was evidence for an asymmetric effect of thermal habitat adaptation as ambient fish transplanted to the geothermal habitat had reduced survival and increased parasite prevalence, while geothermal fish transplanted to the ambient habitat did not, only suffering an increased weight loss. Similar results in the costs of migration have been found for river and lake stickleback, where fish transplanted to either habitat suffered fitness consequences, but in different ways (Kaufmann et al., 2017).

The poorer outcomes for ambient sourced stickleback transplanted to the geothermal habitat may be due, in part, to immunological challenges. The high temperatures of the geothermal habitat better match the thermal optima of *S. solidus*, encouraging its growth and potentially dampening the host's immune system functioning (Franke et al., 2019). Indeed, transplant treatment was found to affect *S. solidus* prevalence with a higher infection rate found in both geothermal and ambient sourced fish in the geothermal habitat. Parasite infection was found to increase weight gain, likely due to the growth of the parasite rather than that of the fish. While it was not possible to establish infection status prior to transplantation, the geothermal habitat may induce greater infection by parasites (possibly there may be a greater abundance of the first-intermediate host (cyclopoida copepods), or an induced change in stickleback foraging strategy that increases consumption of copepods) or facilitate faster parasite growth, either of which is likely to have a fitness consequence (Barber et al., 2004; Franke et al., 2019; Grécias et al., 2018; Heins and Baker, 2003). Such differences in parasite-host dynamics between

habitats could reinforce adaptive divergence and local adaptation (Kaufmann et al., 2017). I also found that ambient sourced fish transplanted to the geothermal habitat were more likely to be infected at the end of the experiment than geothermal native fish. It is unlikely that this difference was simply due to pre-experiment infection status as all fish within the ambient habitat were less likely to be infected than those in the geothermal habitat. Thus, ambient sourced fish in the geothermal habitat are possibly more susceptible to infection, or less able to suppress parasite growth than the geothermal native fish. This may be due to direct differences in immunological systems between geothermal and ambient sourced stickleback, or potentially an additional effect caused by the stress of the geothermal habitat. In either case, this result indicates a fitness consequence for ambient sourced fish transplanted to the geothermal habitat and a sign that geothermal sourced sticklebacks have adapted to their native conditions. However, not all of my findings can be attributed to parasites as after accounting for parasite effects, geothermal sourced fish in their non-native (ambient) habitat are more likely to lose weight than ambient sourced fish in their non-native (geothermal) habitat (Figure 2-3d). This may indicate differences in the stresses of the thermal habitats or potentially locally adapted metabolic differences between fish from different source habitats (Pilakouta et al., 2020).

Asymmetric effects may also arise from the different seasonal temperature variation experienced by the two populations (see Supplementary Table 1). The geothermal and ambient habitats differ in temperature by around 10^{o}C consistently throughout the year; as a result, the winter temperature of the geothermal habitat is close to the summer temperature of the ambient habitat. Therefore, while ambient sourced stickleback transplanted to the geothermal habitat (in a summer experiment) are experiencing a novel temperature, geothermal sourced fish transplanted to the ambient habitat experience temperatures close to what they experience in winter. This suggests that the degree of 'novelty' experienced by the two source populations would differ in my experiment. Indeed, *variation* in weight change (as shown by the spread of the boxplots in Figure 2-3a) was much greater in ambient fish in the geothermal habitat, potentially suggesting that these fish were experiencing a more novel habitat.

Alternatively, asymmetric effects may be caused by evolutionary history. As the geothermal habitat was likely colonised by stickleback originating from the ambient habitat, the transplantation of geothermal sourced stickleback to the ambient habitat represented a return to the ancestral environment. Similarly, reciprocal transplants using chicken (*Gallus gallus domesticus*) populations adapted to different altitudes found asymmetric costs of transplantation, with high-altitude chickens showing little reduction in fitness when transplanted to the ancestral low-altitude habitat (Ho et al., 2020). Gene expression also showed that high-altitude chickens were able to adjust through plasticity to match the native low-altitude profile more closely, while low-altitude chickens in the high-altitude habitat could not. Thus, phenotypic plasticity may play an important role in rapid re-adaptation to ancestral environments by enabling organisms to adapt to a habitat experienced by their ancestors more easily (Parsons et al., 2020; Rajakumar et al., 2012).

2.4.2 Testing relationships between growth and shape

I found evidence supporting prediction 2, that divergent shape between thermal habitats would relate to growth, and prediction 3, that transplanted fish will grow best in the alternate habitat when they possess a body shape similar to native fish. Several aspects of shape from the best performing fish in each transplant group were similar to the typical native shape for each habitat as described by Pilakouta et al. (2023), where geothermal sourced stickleback were found to have deeper bodies, a more subterminal mouth and steeper craniofacial profiles. In my experiment, the ambient habitat appeared to favour a narrower

body depth and a concave profile along the neurocranium (

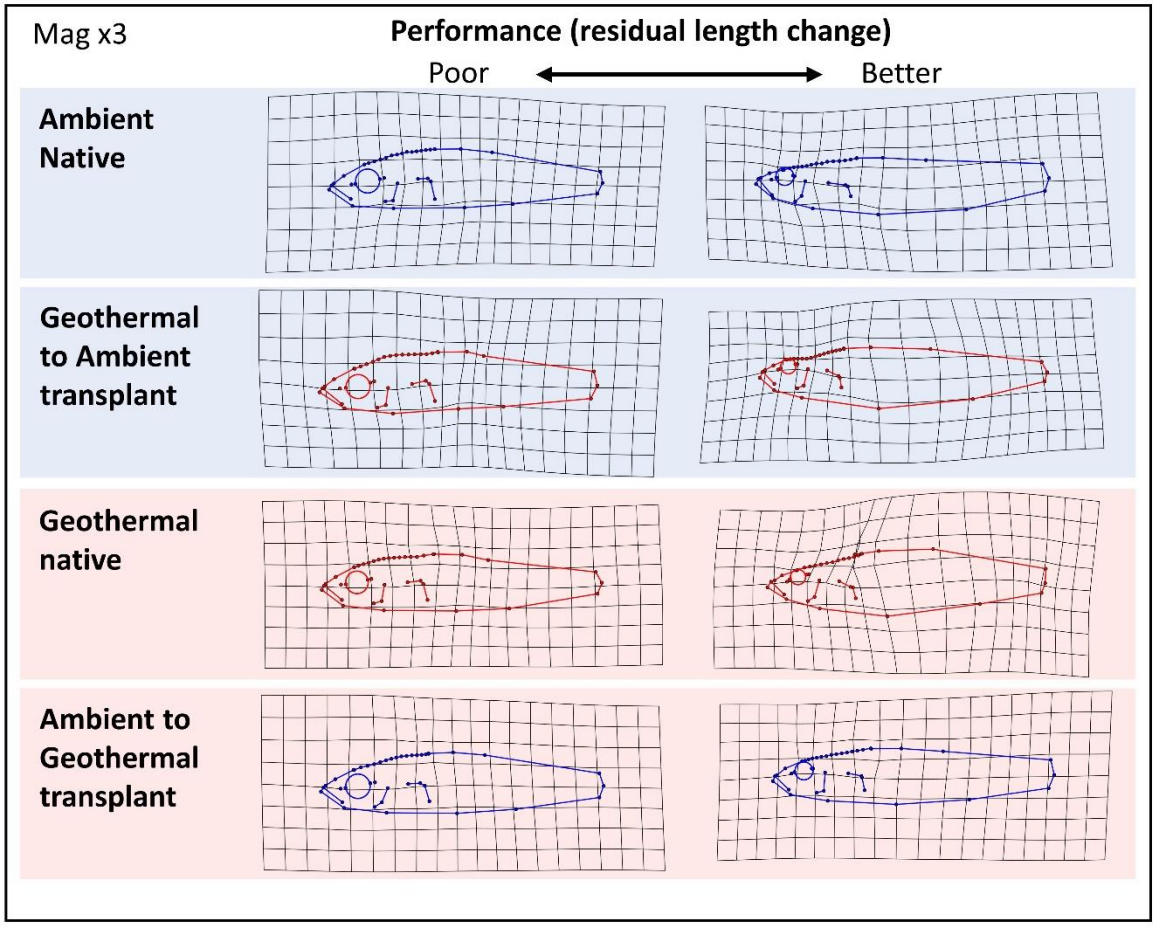


Figure 2-4). This morphology suggested reduced jaw musculature and potentially different foraging and swimming modes relative to the optimum shape in the geothermal habitat (McGee et al., 2013). The geothermal habitat appeared to favour a more subterminal mouth, in line with a shift to a more benthic lifestyle (Table 2-5). (Willacker et al., 2010). These results suggest that the heritable differences in shape between geothermal and ambient stickleback is adaptive in nature. However, while shape was related to growth, the effect sizes were very small (Table 2-3). I note that this was a short-term experiment, and I would not necessarily expect large effects to accrue over a month. Indeed, previous research suggests that even small changes in phenotypic variation can have large fitness effects over a long period. For example, bill shape polymorphism in the African estrildid finch (*Pyrenestes ostrinus*) shows that a difference of less than 1mm in bill length can alter fitness by more than 50% (Smith, 1990). For my experiment I could expect a cumulative effect over a longer period of time, especially during the reproductive season or winter when food availability is low. Further investigation over a different or longer period of

time may therefore reveal more about the potential for stickleback to adapt to thermal habitats.

A more benthic morphology in geothermal habitats could be driven by a shift away from limnetic prey, possibly reflecting differences in prey availability or a dietary change to prioritise higher value prey. Invertebrate communities have been found to differ between geothermal and ambient habitats, with geothermal habitats typically being dominated by large, benthic macroinvertebrates such as gastropods and chironomid larvae (Nelson et al., 2017; O'Gorman et al., 2012; Scrine et al., 2017). Additionally, the geothermal habitat is shallower than the ambient habitat and so the space for a limnetic habitat is reduced. Limnetic prey may therefore be less available to geothermal stickleback, inducing a shift in diet towards benthic prey. This potential dietary shift could also be reflective of the selective pressures of the geothermal habitat, with higher temperatures increasing metabolic rate, and so increasing nutritional requirements and the risk of starvation (Fry, 1967). Indeed, dietary differences and shifts in feeding strategy occur in geothermal populations of brown trout (*Salmo trutta*) that prefer prey that is higher in the trophic web even when such prey items may be relatively rare (O'Gorman et al., 2016). Also, previous research from Áshildarholtsvatn shows that when temperature is increased geothermal source fish experience a smaller increase in metabolic rate relative to their ambient source counterparts (Pilakouta et al., 2020). Therefore, ambient migrants to the geothermal habitat should experience an elevated metabolic rate higher than the native fish increasing the risk of starvation. Dietary shifts between the thermal habitats may also impact *S. solidus* prevalence, as stickleback become infected when they eat an infected copepod, which are typically limnetic. Dietary shifts away from limnetic prey, whether due to abundance or metabolic needs, would therefore be expected to disrupt the route of transmission of the parasite to the stickleback. However, as shown by the results of this experiment, *S. solidus* is particularly prevalent in fish housed in the geothermal habitat, suggesting that geothermal stickleback are indeed consuming infected copepods and so at least part of their diet is made up of limnetic prey.

2.4.3 Testing relationships between growth and allometry

Support for adaptive allometric divergence was in agreement with my fourth prediction. Divergent allometry between geothermal and ambient populations was related to growth, suggesting an adaptive role (Table 2-5 and Appendix 1: Supplementary Figure 2), however, the effect size of this relationship was very small (Table 2-4). This small effect size may have a larger impact in an experiment performed over a longer period of time, or with the inclusion of harsher seasons. Though it is possible that some body shape variation could be attributed to the growth of *S. solidus* distending the abdomen, this would not explain how head shape allometry (a trait which would not be affected by *S. solidus*) interacts with length change (Table 2-4). Diverging allometry in a system which is no older than 70 years may be a surprising result, but some allometric relationships can be quick to evolve (Adams and Nistri, 2010; Bolstad et al., 2015; Frankino et al., 2019; Voje and Hansen, 2013; Voje et al., 2013). It is currently unknown whether this divergent allometry is due to an adaptive plastic response to thermal habitat, or a genetic divergence between geothermal and ambient sourced stickleback. Divergent allometries could partially be driven by plastic temperature effects on growth, with changes in the timing and extent of developmental events being broadly influenced. Thus, while the allometric relationships I have revealed could be due to plasticity, future work examining their heritability will be especially important for determining their evolutionary consequences.

2.4.4 Study limitations.

While a field experiment can be powerful for testing adaptive divergence in a natural setting this approach poses some limitations. For example, the fish used in this experiment were captured in the wild and will have experienced different environmental conditions during development. As such, it is not possible to fully distinguish between differences that are due to heritable divergence, and those that are due to phenotypic plasticity. While there is evidence that the morphological divergence of these fish has a heritable basis (Pilakouta et al., 2023), the differences in survival and parasite prevalence found in this experiment may be related to either or both genetic divergence or phenotypically plastic shifts.

Additionally, the density of free-living stickleback was unknown; thus, the experimental density I used may have impacted my results. Indeed, it is possible that fish density differs between geothermal and ambient habitats, making one type better adapted to higher levels of competition. For this experiment I selected equal densities of 25 sticklebacks per cubic metre across all cages. This value was selected as it is within the range of previous reciprocal transplant experiments involving this species, which ranges from approximately 0.05 sticklebacks per m^3 used by Räsänen and Hendry (2014) to approximately 133 sticklebacks per m^3 used by Kaufmann et al. (2017). Additionally, the fish used in this experiment were not yet full sized and so a higher density was possible.

Due to the shallow nature of the geothermal habitat, cage dimensions were selected for equal volumes between habitat, however, this results in more water surface area available to fish in geothermal habitat cages which may impact their ability to forage benthically compared to fish housed in ambient habitat cages. Such a challenge is common in reciprocal transplant experiments, and differing dimensions of enclosures are often used (Hendry et al., 2002; Räsänen and Hendry, 2014; Stutz et al., 2015).

Predation was also prevented but could be relevant for a fish migrating to a different thermal habitat. I also expect that predation would differ between thermal habitats, as piscivorous birds may prefer hunting in geothermal water (Esler, 1992; Stocking et al., 2018), while salmonid predators would likely avoid them (Santiago et al., 2016). Indeed, Arctic terns (*Sterna paradisaea*) were frequently observed catching fish in the geothermal habitat, which is shallower than the ambient habitat. Also, while each cage was provided with mud and native plants, exactly matching the natural variation between wild habitats would be difficult to achieve in an artificial cage, particularly for the ambient habitat, which was larger and deeper and potentially more variable. The complexity of a habitat can affect the optimal shape, for example, a more densely planted and complicated habitat could benefit a deeper body more adept at manoeuvring (Domenici et al., 2008). As my cages could not exactly replicate the natural environment in terms of vegetation and predation, it is possible that this experiment will not represent the full nature of this study system. A further limitation of this experiment was the limited data collected on parasite infection. As this was only a preliminary investigation, only infection

status was collected and so the size and number of *S. solidus* was not known. As I have shown a significant impact of the thermal habitat on host-parasite dynamics in this system, and the potential for worse outcomes for non-native fish in the geothermal habitat, further study of *S. solidus* and its host in this study system would be beneficial. Finally, this geothermal system differs from climate change driven temperature increases in that temperature change would have occurred rapidly rather than gradually. Gradual environmental change can result in delayed evolution, due to weaker selective pressure and the potential for phenotypes selected for partway through the process of environmental change to prove to be dead ends, not useful at more extreme degrees of environmental change (Gorter et al., 2015; Guzella et al., 2018). However, adaptation to different rates of environmental change can still result in similar fitness endpoints (Gorter et al., 2015).

2.4.5 Conclusions

My findings suggest that stickleback found in geothermally warmed system have adapted to their habitat. Therefore, while increasing temperatures will pose challenges it may be that species with a tendency for phenotypic change could be more persistent under climate change. Geothermal sourced stickleback suggests a shift to a benthic lifestyle through morphology, with contributions from allometric variation, but also a potential change in immunity. This is notable given that the geothermal habitat has been established for no more than 70 years. The underlying mechanisms for such changes are likely to be insightful for other populations as a number of traits in fish are known to be plastic in response to temperature (Campbell et al., 2021; Crozier and Hutchings, 2014; Sfakianakis et al., 2011), but it is also the case that adaptive evolutionary divergence in fish can be extremely rapid (Barrett et al., 2011; Kovach et al., 2012) with evidence of heritable divergence in this system (Pilakouta et al., 2023). I suspect that a wide range of factors contribute toward adaptive divergence between thermal habitats, and it will be an important challenge to discern how they contribute to adaptive divergence in a warming world.

3 Chapter 3: Divergent gene expression and thermal plasticity in geothermal, ambient and hybrid stickleback

3.1 Introduction

Climate change driven increases in temperature will present an ever-worsening threat to animal populations (IPCC, 2021). Ectotherms are expected to be particularly vulnerable to temperature changes due to their limited ability to directly thermoregulate (Fry, 1967; Paaijmans et al., 2013). Facing novel temperatures, and the accompanying changes in ecological conditions, ectotherms will therefore need to migrate away from or rapidly adapt to survive. Climate change has already been implicated as the cause of adaptive phenotypic shifts across fishes (Crozier and Hutchings, 2014). However, the mechanisms underlying such changes are generally unclear with a heritable basis being untested in most cases, but with phenotypically plastic responses being demonstrated more commonly (Crozier and Hutchings, 2014). It is increasingly important to understand how climate change adaptation could involve interactions between plasticity and genetic variation. Determining the mechanisms of such change is an area of growing interest as it can inform conservation management strategies. Thus, conservation efforts should leverage more recent concepts in evolutionary theory that incorporate the range of influences on phenotypic development to predict how climate change will impact biodiversity (Campbell et al., 2017; Parsons, 2021; Rilov et al., 2019).

The concept of evolvability can provide a framework that brings together a range of disciplines, including concepts of evolutionary genetics and phenotypic plasticity. Evolvability focuses on how populations can generate adaptive variation regardless of whether it is genetically-based, plastic, or from a combination of the two (Campbell et al., 2017; Pigliucci, 2008; Riederer et al., 2022). Evolvability is relevant to a changing environment, and can involve relationships between genetic and phenotypic variation that can be broadly described as genomic, regulatory, and environmental effects (Hansen, 2006; Riederer et al., 2022). Genomic effects can be additive, whereby two alleles contribute equally to the phenotype, or non-additive whereby dominance, or epistasis can be attributed to trait variance (Falconer and Mackay, 1996). Complementing this, regulatory effects can be broadly divided into trans- or cis-regulatory elements (Wittkopp et al., 2004). Cis-regulation occurs via non-coding

DNA close to the controlled gene (i.e. promoters, silencers and enhancers) while trans-regulation is performed by factors distant to the gene (i.e. transcription factors and noncoding RNAs) (Wittkopp et al., 2004; Zhong et al., 2019). Cis-regulatory processes have tended to underlie evolutionary divergence in gene expression more often than trans-regulatory changes (Signor and Nuzhdin, 2018; Verta and Jones, 2019; Wittkopp et al., 2004; Zhong et al., 2019). A potential reason for this may be due to the different relationships between cis- and trans-regulatory variation and genomic effects. Cis-regulatory variation is more often associated with an additive effect in hybrids and so is more effectively acted upon by selection (Lemos et al., 2008). However, cases exist where trans-regulatory mechanisms contribute more to adaptive divergence, such as in *Cyprinodon* pupfishes, where the majority of expression divergence between species was explained by trans-regulatory mechanisms (McGirr and Martin, 2021).

Environmental effects can strongly impact gene expression, and can include interactions with additive effects, and evidence also suggests that cis- and trans-regulatory elements can be influenced by the environment (Li and Fay, 2017). Such plasticity in regulatory elements may play an important role in evolvability (Campbell et al., 2017), especially in light of climate change. Indeed, plastic responses could allow a population to persist in new conditions, and in some cases buy time for genetic change (Crispo, 2008). However, plasticity and genetic change are often intertwined as plasticity itself can be responsible for the release of adaptive cryptic genetic variation (Ghalambor et al., 2007). The refinement of such cryptic variation under specific environments can provide a leading edge for phenotypic divergence (Ho et al., 2020; Parsons et al., 2020). In line with this the 'plasticity-first' hypothesis suggests that phenotypic plasticity can aid evolution when a novel environment induces a plastic response that can then be refined and, in some cases, canalized as genetic change (Levis and Pfennig, 2019b, 2019a). Indeed, while many of these processes have been posited, an opportunity exists to directly investigate the specific regulatory and gene expression changes that can occur across environments in the context of climate change.

Climate change has been implicated in geographic range shifts (Al-Chokhachy et al., 2013; Crozier and Hutchings, 2014; Rahel and Olden, 2008;

Wenger and Olden, 2012), which are likely to alter gene flow between locally adapted populations (Pauls et al., 2013). This could be especially problematic for populations spread over wide-ranging environmental clines, or inhabiting different microhabitats, as they can display extremely different local adaptations (Kawecki and Ebert, 2004). Partial reproductive isolation between locally adapted populations can form rapidly, within 10-20 generations in some cases in vertebrates(Hendry et al., 2007), and in invertebrates, reproductive isolation may form within mere years (Hendry et al., 2007; Villa et al., 2019). Full reproductive isolation, where offspring are inviable or infertile, takes longer to form (Kulmuni et al., 2020). Locally adapted populations with newly overlapping ranges are therefore unlikely to face full reproductive barriers, and so gene flow, with partial reproductive barriers, is possible. If viable and fertile hybrid offspring are produced between divergent populations, then it is unlikely their phenotypes will match either parental niche (Gow et al., 2007; Rundle and Nosil, 2005). Hybridization can often produce intermediate phenotypes, as well as transgressive phenotypes, whereby the hybrid phenotypes exceeds the range of the phenotype seen in either parent (Landry et al., 2005; Maheshwari and Barbash, 2011; Rieseberg et al., 1999). Hybrids are also likely to suffer consequences from a disruption of adaptive gene by environment interactions. (Carroll et al., 2003; Cenzer, 2017). Indeed, if parental populations have diverged in the nature, direction or strength of plastic responses, then hybrids should inherit a mix of the genetic components underlying each. Therefore, addressing how parental and hybrid offspring of thermally adapted populations respond to temperature variation could be particularly insightful for understanding how genomic and regulatory mechanisms might be impacted by climate change.

Systems in the wild that can address how the thermal habitat impacts divergence and evolvability are rare. Often this involves comparisons of populations from widely varying latitudes or altitudes, but this means other factors can be confounded. However, some populations of three-spined stickleback (*Gasterosteus aculeatus*) have been found inhabiting adjacent geothermal and ambient temperature habitats across Iceland (Millet et al., 2013; Pilakouta et al., 2020, 2023). This creates a strong thermal gradient that typically differs by around 10^{o}C throughout the year, although at some sites this temperature difference may be as much as 21^{o}C during the winter (Pilakouta et

al., 2020). Some of these populations, where geothermal and ambient sites are in very close proximity, show evidence of heritable adaptive divergence even in populations as young as 70 years (unpublished data, Pilakouta et al., 2023). Here I focus on one of these geothermal-ambient pairs because it provides an opportunity to examine the effects of temperature induced plasticity, habitat divergence and hybridization. Specifically, using lab rearing experiments on fish derived from a geothermal/ambient pair I focus on patterns of gene expression between geothermal and ambient sticklebacks, its response to rearing temperature and its disruption in hybrids.

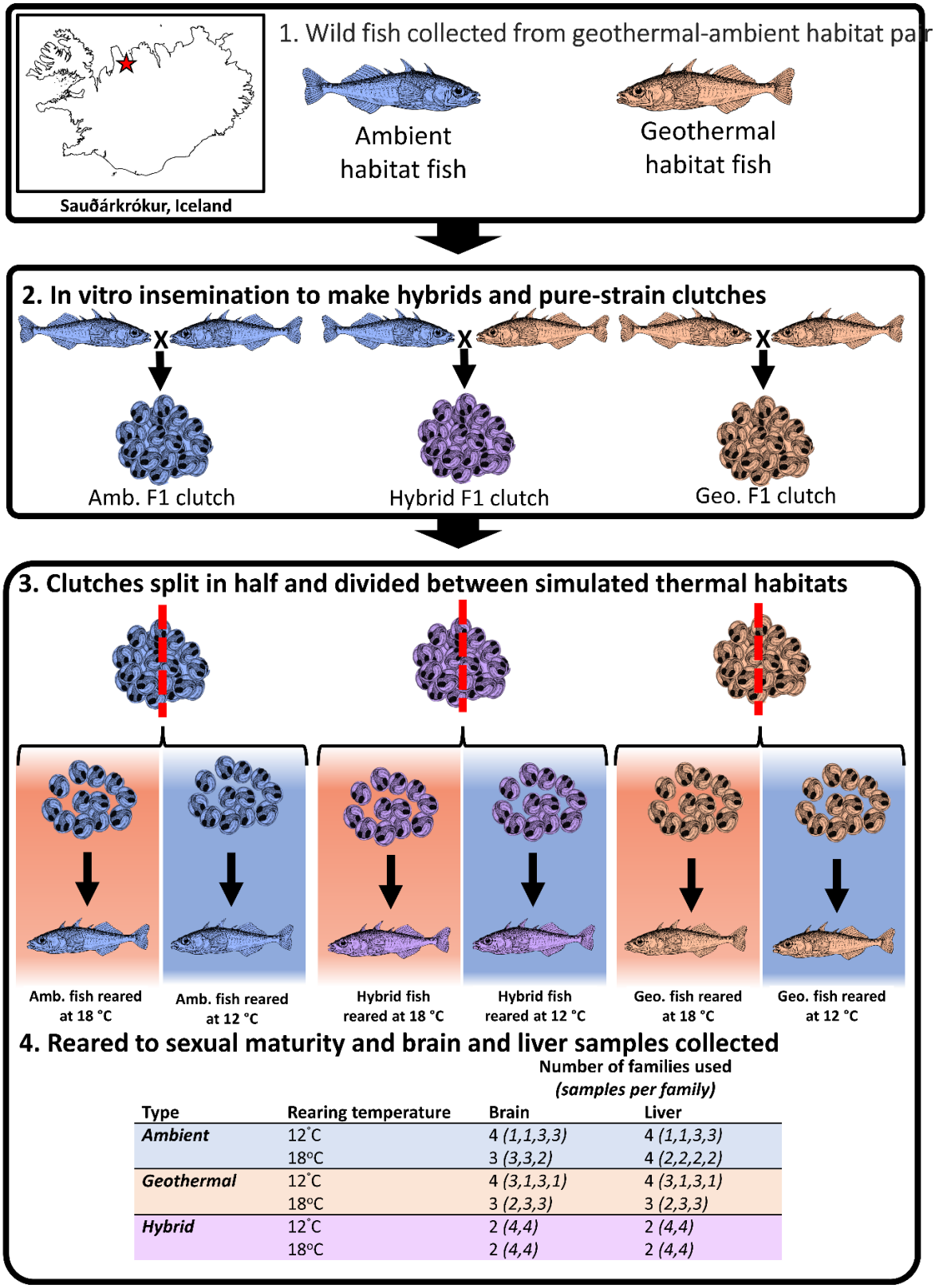
I predicted 1) that sticklebacks from different thermal habitats would show divergence in gene expression, gene co-expression and plastic responses to rearing temperature. I also predicted 2) that gene expression of hybrids would differ from pure-strain (geothermal and ambient non-hybrids) gene expression, and that hybridization would result in the disruption of gene interactions relative to pure-strain fish. Regarding inheritance mechanisms, I predicted 3) that given rapid evolutionary divergence (Pilakouta et al. 2023), divergent gene expression between geothermal and ambient sticklebacks would be contributed to by primarily by additive variation, as additive variation is key to heritability and the evolutionary potential of a trait (Falconer and Mackay, 1996). Finally, I predicted 4) that geothermal and ambient fish have diverged in regulatory mechanisms, that will result in transgressive hybrid gene expression (Go and Civetta, 2020), and that cis- effects will be more prominent than trans effects as expected for microevolutionary processes (Signor and Nuzhdin, 2018; Verta and Jones, 2019; Wittkopp et al., 2004; Zhong et al., 2019).

3.2 Methods

3.2.1 Creation of F1 hybrid and pure strain families.

Sexually mature adult sticklebacks were caught using un-baited minnow traps (mesh size, 6.4mm) from an allopatric geothermal-ambient habitat pair in Northern Iceland, near Sauðárkrókur (geothermal habitat: 65.732260º, -19.618937º, ambient habitat 65.732191º, -19.618574º) in 2016 and brought to the University of Glasgow. Ambient and geothermal summer temperatures were simulated in the aquaria using 12°C (±1°C) and 18°C (±1°C), respectively. Fish were primed for reproduction with nutrient rich food (bloodworms, mysis) and an extended light period (20-24hrs). In vitro fertilisation was performed to

create independent families following Barber and Arnott (2000). Male sticklebacks were euthanised and sperm extracted from the gonadal tissue through maceration in a sterilised container. Gravid female sticklebacks were stripped of eggs into a moistened petri dish through the application of gentle pressure to the abdomen. The macerated gonadal tissue of the male was then added and mixed with a paintbrush. The petri dishes containing the egg clutches were then covered with a lid and left to fertilise for fifteen minutes before being split evenly in half. Each half went into one of two mimicked thermal habitats (12^{o}C representing the ambient habitat and 18^{o}C representing the geothermal habitat). This included four ambient habitat, and four geothermal habitat sourced families, where both parents originated from the same thermal habitat, and two hybrid families created using two SKR geothermal females crossed with two SKR ambient males. Each egg clutch was split evenly in half immediately after fertilisation and divided between the two temperatures simulating ambient and geothermal habitats (12°C (±1°C) and 18°C (±1°C)) to create six treatment types (Figure 3-1).



Type	Rearing temperature	Number of families used (samples per family) Brain	Liver
Ambient	12°C	4 *(1,1,3,3)*	4 *(1,1,3,3)*
	18°C	3 *(3,3,2)*	4 *(2,2,2,2)*
Geothermal	12°C	4 *(3,1,3,1)*	4 *(3,1,3,1)*
	18°C	3 *(2,3,3)*	3 *(2,3,3)*
Hybrid	12°C	2 *(4,4)*	2 *(4,4)*
	18°C	2 *(4,4)*	2 *(4,4)*

Figure 3-1 Experimental design showing collection of sticklebacks and subsequent creation of F1 pure-strain and hybrid fish.

Fertilized egg clutches were kept in mesh baskets submerged within larger basins of well-aerated water fed by a recirculated continuous water drip system. Water was treated with methylene blue (2.5 µg/ml) until hatching to reduce the risk of fungal infection. Containers were inspected daily, and dead or infected eggs were removed immediately. After hatching, larvae and juveniles were fed live food (newly hatched brine shrimp (*Artemia* salina), and microworms (*Panagrellus redivivus*) as well as size-appropriate ZM powdered food (ZM100 and ZM200) (ZMsystems, Twyford, UK). Upon reaching approximately 2cm, juveniles were moved, in their family groups, to 10L tanks at standardized densities of 15-20 individuals. At maturation the fish were fed trout pellets (Microstart, EWOS Ltd, Surrey, UK). Temperatures were monitored and recorded daily and water chemistry parameters (ammonia, nitrite, nitrate, TPM and pH) were tested twice weekly and adjusted as necessary. The photoperiod of the aquarium simulated that of their natural habitat in Iceland, including seasonal changes in photoperiod.

3.2.2 Tissue collection, RNA extraction and sequencing

Fish were sacrificed between October and December of 2018 at sexual maturation using an overdose of benzocaine and subsequent confirmation of death via cervical dislocation. To limit contamination work surfaces and dissection tools were cleaned before and after each organ dissection using RNAseZap solution (AM9780, Thermo Fisher Scientific, Massachusetts, US). Fish were sexed by the identification of testes or ovaries, and I aimed to sample even sex ratios across treatment groups. For each fish whole brain and livers were removed, cut into pieces smaller than 0.5cm^3 and stored separately in RNAlater at room temperature overnight before freezing at -80°C. I collected eight samples per tissue within each of the six treatments, resulting in a total of 96 samples (48 brains and 48 livers). Samples were selected from 3-4 families in geothermal and ambient fish, and 2 families of hybrid fish (see Figure 3-1 for sample distribution across families).

To obtain RNA, extractions were performed using a Qiagen RNeasy standard kit (Qiagen Cat ID: 74104) for liver tissue (with 50% ethanol as recommended for fatty tissue such as liver) and the Qiagen RNeasy lipid tissue kit (Qiagen Cat ID: 74804) for brain tissue (as this kit was found to work best with this tissue). Approximately 20mg of tissue was used for each extraction.

RNA purity was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, US). All samples were required to have a 260/230 or 260/280 ratios above 1.8, thus in some instances ethanol precipitation or re-extraction were used. RNA concentration was determined using a Qubit 4 Fluorometer using a Qubit RNA Broad Range kit (Thermo Fisher Scientific, Massachusetts, US). Tissue samples where the extracted RNA showed low concentrations (below 100ng/ul) were re-extracted from the same sample, if enough tissue remained, or a suitable replacement (a sample from the same treatment group). RNA integrity was assessed for all samples using an Agilent 4200 Tapestation machine (Agilent Technologies, California, US) to calculate the RNA integrity number (RIN values), a numerical score ranging from 1-10 where a higher value indicates a higher degree of RNA integrity (Sheng et al., 2017). Samples with RIN values below 7 were re-extracted or replaced and re-tested. Two liver samples (within two families in the pure-strain ambient reared at 18°C group), required re-extraction, and due to limited tissue, were replaced with livers collected from two additional individuals (from a third family in the same treatment group).

The preparation of cDNA libraries and whole transcriptome sequencing were performed by the CGR, The University of Liverpool using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB E7490) and NEBNext® Ultra RNA Library Prep Kit for Illumina® (E7530). Sequencing was performed on the Novaseq platform (Illumina, California, US). I aimed for an average read length of 150 base pairs and 20M sequenced reads per sample.

3.2.3 RNA-seq data analysis

3.2.3.1 Initial data processing and quality control

I verified read quality with *FastQC* (Andrews, 2010) and *MultiQC* (Ewels et al., 2016) software. However, first the obtained Fastq files were trimmed for the presence of Illumina adapter sequences using *Cutadapt* version 1.2.1 (Martin, 2011) and then further trimmed using *Sickle* version 1.200 (Joshi and Fass, 2011). RNA-seq reads were then aligned to version five of the three-spined stickleback reference genome (Genbank GCA_016920845.1) (Peichel et al., 2020) using the *Hisat2* aligner version 2.2.0 (Kim et al., 2019). The resulting SAM files, which contained the alignment information for each sample, were then sorted and converted to BAM format, compressed binary versions of SAM files, using

Samtools sort and indexed using *Samtools index* (Danecek et al., 2021). Read counts were obtained using *Htsqcount2* (Anders et al., 2015).

3.2.4 Differential expression analysis between ecotypes and temperatures

Data analysis was performed in R (v4.3.0) (R Core Team, 2022). Read counts were analyzed using both *EdgeR* (Robinson et al., 2009) and *LIMMA* (Ritchie et al., 2015). *EdgeR* and *LIMMA* were used in parallel to provide a high confidence in genes identified as differentially expressed in both analyses.

3.2.4.1 Pre-processing of raw counts

Read count data was pre-processed with filtration for low-expressed genes using *filterbyExpr*. Genes were removed from the analysis if they had fewer than ten counts-per-million (CPM) in more than 8 samples in the brain and 8 samples in the liver (the sample size of the smallest group for each dataset) (Chen et al., 2016). Read counts were normalized to prepare for downstream analysis. Normalization was performed by converting observed library sizes into effective library sizes using scaling factors calculated by *calcNormFactors* using the TMM method (Robinson and Oshlack, 2010). I also tested for overall differences in gene expression variance between treatment, and tissue types using a Levene's test from the *leveneTest* function in the *car* package (Fox and Weisberg 2019). Levene's tests were performed on a gene-by-gene basis following methodology from Le Priol et al. (2023).

3.2.4.2 Modelling of count data

To model the effects of fish type and rearing temperature on gene expression I used a design model matrix with an interaction between fish type (geothermal pure-strain, ambient pure-strain, or hybrid), and rearing temperature (12°C or 18°C), with sex as a covariate. I used the same design model matrix for both *EdgeR* and *LIMMA* analyses. Gene-wise models were then fitted to the count data using the design model matrices.

In *EdgeR* a negative binomial generalized linear model was fitted to each gene. This analysis assumed that the variance of gene counts is dependent on the negative binomial dispersion and the quasi-likelihood dispersion (Ren and Kuan, 2020). The negative binomial dispersion represents the variability of the biological system, while the quasi-likelihood dispersion represents gene-specific variability greater or lower than the overall level, capturing both biological and technical sources of variability (Lun et al., 2016). *EdgeR's estimateDisp* function

was used to fit a mean-dispersion trend across all genes in the dataset to estimate the negative binomial dispersion (Lun et al., 2016; Ren and Kuan, 2020). An empirical Bayes approach was then used to estimate the quasi-likelihood dispersion and a generalized linear model accommodating the design model matrix was fitted using *glmQLFit* (Lun et al., 2016; Ren and Kuan, 2020). This method uses the quasi-likelihood (QL) methods developed by Lund et al., (2012) to account for gene-specific variation from biological and technical sources (Lun et al., 2016).

Normalized read counts were prepared for linear modelling in *LIMMA* by performing a voom transformation. The voom method incorporates the mean-variance relationship of the log-counts into the precision weights for each observation (Law et al., 2014, 2018). Next, a linear model was fitted to each gene using *lmFit* (Law et al., 2018).

3.2.4.3 Design of contrasts to test experimental questions.

The questions of this experiment were then addressed by applying tests to the fitted models using contrasts designed to answer each question. Contrasts describe the linear combinations of parameters that should be used to calculate the differences between groups of interest (Law et al., 2020). For example, a contrast of A – B would calculate the difference in means between A and B.

To test for divergence between geothermal and ambient sticklebacks (prediction 1), a contrast matrix where geothermal fish gene expression at a given rearing temperature was subtracted from ambient gene expression at the same temperature, resulting in two contrasts (“*ambient at 12˚C – geothermal at 12˚C”* and “*ambient at 18˚C – geothermal at 18˚C”*) was used. To further examine divergence in terms of geothermal and ambient stickleback plasticity (prediction 1) I used a contrast matrix where pure-strain gene expression at 18˚C was contrasted against the same pure-strain gene expression at 12˚C, resulting in two contrasts (“*geothermal at 12˚C – geothermal at 18˚C”* and “*ambient at 12˚C – ambient at 18˚C”)*. Genes found to be DE between rearing temperatures were considered to be plastic.

Testing for a mismatch between hybrids and pure-strain fish (prediction 2), used a contrast matrix where hybrid gene expression at each temperature was subtracted from a given pure-strain gene expression at the same temperature, resulting in four contrasts (“*ambient at 12˚C – hybrid at 12˚C”,*

"ambient at 18°C – hybrid at 18°C", "geothermal at 18°C – hybrid at 18°C", and *"geothermal at 12°C – hybrid at 12°C"*). Hybrid plasticity was separately addressed using a contrast matrix where hybrid gene expression at 18°C was subtracted from hybrid gene expression at 12°C (*"hybrid at 12°C – hybrid at 18°C")*.

3.2.4.4 Hypothesis testing

In *EdgeR*, DE in each contrast was tested for each gene in the model using quasi-likelihood F-tests (using the function *glmQLFTest)* (Lun et al., 2016). The top-ranked genes for each contrast were then extracted using *TopTags*. In *LIMMA*, DE in each contrast was tested for each gene in the model by using *eBayes* to perform a moderated F-statistic test and rank genes by evidence of DE for each contrast (Law et al., 2018). The top-ranked genes for each contrast were then extracted using *topTable*. For all of these analyses the false discovery rate (FDR) was controlled using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). This was done in *EdgeR* and *LIMMA* during the extraction of significantly DE genes using *TopTags* and *topTable* respectively. Genes were considered to be differentially expressed (DE) when the resulting adjusted p value was below 0.05. I assessed the reliably of both *EdgeR* and *LIMMA* analysis methods by testing for a correlation between the results of each method. I assessed whether *EdgeR* and *LIMMA* methods assigned similar levels of significance to the DE of each gene in each analysis by testing the correlation between the *EdgeR* and *LIMMA* adjusted p values for each gene. This correlation test was performed with a Pearson's correlation using *cor* from the *Stats* R packages (R Core Team, 2022). After ensuring that the *EdgeR* and *LIMMA* results correlated with each other, I selected only genes that were found to have significant DE in both *EdgeR* and *LIMMA* analyses for further analysis. Each question was then addressed by assessing the intersection of divergent genes in each pairwise contrast.

Divergence that is robust to rearing temperature may suggest genetic divergence, or divergent transgenerational effects (Bateson and Gluckman, 2012). I selected genes exhibiting robust divergence between geothermal and ambient pure-strain fish was assessed by intersecting genes that were DE at 12°C and 18°C. Genes that were DE at both 12°C and 18°C, and with DE in the same

direction (e.g., upregulated in geothermal fish) at both rearing temperatures, were considered to be evidence of robust divergence.

Divergent geothermal and ambient plastic responses within pure-strain fish was assessed by finding intersecting genes that were plastic in geothermal fish with genes that were plastic in ambient fish. Genes that were plastic in both geothermal and ambient pure-strain fish were considered to be a part of a common plastic response to rearing temperature. Genes that were plastic in either the geothermal or the ambient pure-strain fish, but not in both, were considered to be evidence of potentially divergent plastic responses.

3.2.5 Testing for divergence and disruption in gene co-expression using WGCNA network analysis.

The expression of a gene does not occur in isolation and it has been found that genes often co-express together in a network of gene clusters (Wen et al., 1998). The co-expression of genes suggests that the genes within a module share a biological relevance to each other (Oldham et al., 2006). Changes in gene module networks between populations can be used to discover key drivers of evolutionary divergence (Oldham et al., 2006). Furthermore, hybrids of divergent populations have been found to display disrupted co-expression networks, potentially contributing to reproductive isolation (Filteau et al., 2013). To identify divergence in the interactions of genes between geothermal and ambient fish (prediction 1) I first identified modules of genes with similar patterns in expression within geothermal and ambient fish, and then tested for the preservation of modules between geothermal and ambient fish. I also tested for the disruption of modules in hybrid fish (prediction 2) by also identifying gene modules within hybrids and testing for module preservation between hybrids and each pure-strain type.

3.2.5.1 Identification of gene modules

First, I identified modules of genes with similar patterns of expression using a weighted correlation network analysis (WGCNA) using the R package *WGCNA* (Langfelder and Horvath, 2008). This method uses hierarchical clustering to group similarly expressed genes and assign them to modules. For this analysis I used a soft thresholding power, selected by the criterion of approximate scale-free topology using the function *pickSoftThreshold* as per Zhang and Horvath (2005), to reduce the noise of the correlations in the network. Next, the

network adjacency was calculated using *adjacency* and hierarchical clustering was performed to produce a hierarchical clustering tree of genes using *hclust*. Clusters of genes (modules) were then identified from this cluster tree using *cutreeDynamic*, with a minimum module size of 30 genes. Modules with very similar expression profiles were merged together based on the first principal component of each module (the module eigengene), calculated using *moduleEigengenes*. The dissimilarity of the module eigengenes was calculated using *cor* and modules with correlation of 0.75 or higher were merged using *mergeCloseModules*.

3.2.5.2 Testing for divergence and disruption in module networks

In order to assess divergence in gene interactions between geothermal and ambient fish (prediction 1), and disruption of gene modules in hybrids (prediction 2), I ran an analysis of the preservation of modules between groups. Module preservation between geothermal and ambient fish, hybrids and geothermal fish and hybrids and ambient fish, was calculated using the *modulePreservation* function (*WGCNA* package, 200 permutations (Langfelder and Horvath, 2010)). This function uses the density and pattern of connections within modules and between datasets to generate $Z_{summary}$ scores. A strong module preservation is indicated by a $Z_{summary}$ score of ≥10, a weak module preservation is indicated by a $Z_{summary}$ score of between 2 and 10, and a lack of module preservation is indicated by a $Z_{summary}$ score <2 (Langfelder et al., 2011). Modules with no preservation between ambient and geothermal fish were considered to be evidence of divergence in gene interactions. Hybrid modules with no preservation to either geothermal or ambient fish were considered to be evidence of a disruption to gene interactions in hybrids in the pure-strain to hybrid comparisons.

3.2.5.3 Testing for divergence and disruption in module plasticity

Additionally, plastic responses of geothermal, ambient and hybrid fish were assessed for modules (predictions 1 and 2). Modules involved in the plastic response to temperature were tested for using Gaussian GLMs to examine the effect of rearing temperature on module eigengene values, with sex as a covariate. Modules with significant relationships to rearing temperature were selected for gene ontology analysis.

In order to assess whether module plasticity was conserved between groups, a consensus network was created from geothermal, ambient, and hybrid data sets by calculating a consensus topological overlap across all three groups. Clustering, module assignment, and the merging of similar modules was then carried out using the same method used for the individual group networks. Each group's network was then compared to the consensus network by calculating the overlaps of each pair of modules, and a Fisher's exact test was used to calculate the relatedness of each module pair. Modules from one network may be related to the modules of another network via consensus modules by examining the resulting module correspondence tables. A module in one network may correspond to more than one module in another network. Pure-strain modules found to be plastic in one type of pure-strain fish but lacking corresponding plastic module(s) in the other pure-strain fish were considered to have divergent plasticity (prediction 1). Hybrid modules found to be plastic but lacking a corresponding plastic module in pure-strain fish, and pure-strain plastic modules that lacked a corresponding plastic module in hybrid fish were considered evidence of disrupted plasticity (prediction 2).

3.2.6 Hybrid gene expression and inheritance mode categorization

To address prediction 3) it was necessary to categorise the inheritance mode of gene expression. Therefore, in each rearing temperature I compared hybrid expression relative to pure strain fish. Genes with DE in at least one of the previous hybrid to pure-strain contrasts were selected for comparison, and inheritance modes were classified separately for each rearing temperature. Specifically, genes were categorised based on the log fold changes (LFC) of DE between hybrids and each pure-strain type with a threshold of 0.32 (1.25-fold difference, shown to be a useable cut-off by Wang et al., (2022) and Yazdi et al., (2022). DE was categorised as additive, dominant, or transgressive, (Figure 3-2) on the basis of the LFC threshold. Additive DE was classified as hybrid gene expression 0.32 LFC higher than one pure-strain type and 0.32 LFC lower than the other pure-strain type. Dominance was classified as hybrid gene expression within 0.32 LFC of one pure-strain type, but not the other. Finally, transgressive DE was identified as hybrid gene expression 0.32 LFC higher or lower than *both* pure-strain types.

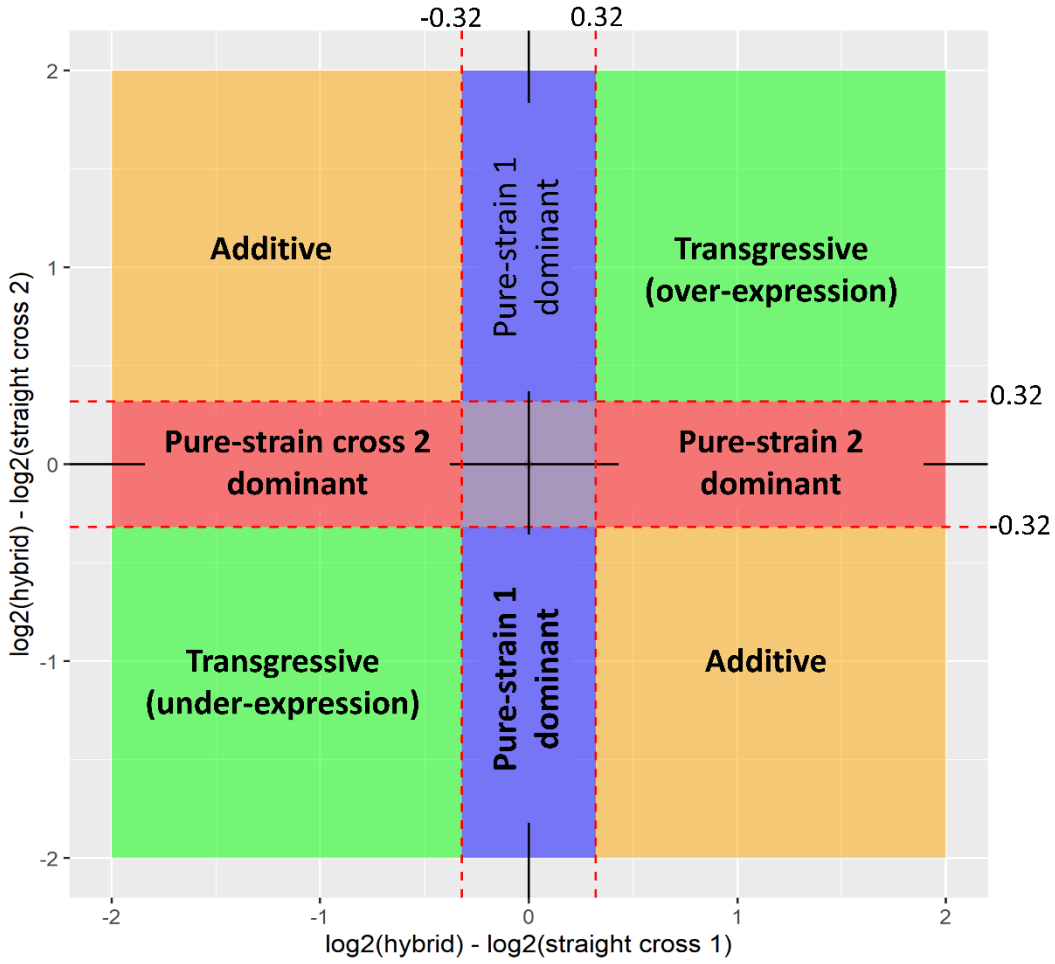


Figure 3-2 Classification of hybrid gene expression inheritance type via cut-offs of differential expression when contrasted with pure-strain types (adapted from (Yazdi et al., 2022)*)*

3.2.7 Comparing ecotype regulatory mechanisms through Allele specific expression analysis

Divergence in regulatory mechanisms (prediction 4) was assessed through allele-specific expression in F1 hybrids, which should correspond to cis-regulatory change. The premise for this is that one copy of each pure-strain chromosome should be present in each cell of an F1 hybrid, meaning that while the trans-regulatory environment for each allele is the same, the cis-regulatory environment may differ. Differences in cis-regulatory elements can result in the differential expression of each allele in the F1 hybrids, which is detected as allele-specific expression.

To assess the presence of allele specific expression, aligned Bam files were prepared for variant calling using the *GATK* best practices RNAseq short

variant discovery workflow (Brouard et al., 2019; Caetano-Anolles, 2023b). Post-alignment quality was checked using *Picard ValidateSamFile*. Mate-pair information was verified and fixed, and duplicate reads tagged using *Picard's FixMateInformation* and *MarkDuplicates* (Broad Institute, 2023). *GATK's SplitNCigarReads* was used to reformat alignments spanning introns for compatibility with *HaplotypeCaller*. An initial round of variant calling was performed in order to bootstrap a set of known variants, which were filtered (*GATK: VariantFiltration, SelectVariants*) using hard filters (Quality by Depth (QD) <2.0, Fisher Strand (FS) > 60, RMS Mapping Quality (MQ) < 40, Strand Odds Ratio (SOR) >4, as recommended by *GATK* best practices (Caetano-Anolles, 2023a)), for input into *GATK's Base Quality Score Recalibration*. Base quality recalibration was performed using machine learning applied to each sample to detect and correct patterns of systematic errors in the base quality scores using *BaseRecalibrator*. A second round of variant calling using *HaplotypeCaller* provided variants, which were also filtered (*VariantFiltration, SelectVariants*) using hard filters (QD <2.0, FS > 60, MQ < 40, SOR >4). SNPs were called for all fish (geothermal, ambient and hybrid). Pure-strain SNPs were filtered for SNPs that were homozygous across each type (geothermal or ambient). SNPs unique to geothermal and ambient types were then extracted.

SNPs identified in hybrid fish were filtered to include only those that were heterozygous across all hybrids in order to target SNPs that may be habitat divergent. Homozygous SNPs were excluded from the analysis. Allele specific reads were then output from the VCF files using GATK's *ASEReadCounter*. SNPs were mapped to genes using *SNPeff*. SNPs were filtered for a total read count of >20, a minimum read count for the reference and alternatives of >5, and SNPs were only used for this analysis when present and passing filters for all fish within a treatment group.

Allele specific expression was then assessed per SNP using Allele-Specific Expression analysis in a Population (ASEP) (Fan et al., 2020). ASEP is a method for gene-level detection of allele-specific expression at the population level without individual genomic data, using a pseudo-phasing procedure that uses allele-specific read counts to infer haplotype phases through a majority voting procedure based on allele specific read counts. A generalized linear mixed-effects model is then used to test for allele-specific expression. Because

differing levels of allele-specific expression between rearing temperatures could indicate environmental effects on expression regulation ASEP analysis was performed separately for hybrids reared at 12°C and 18°C. Furthermore, to assess the biological relevance SNPs identified by ASEP analysis were cross-referenced to SNPs present in pure-strain fish that were unique to either the geothermal or ambient fish types.

3.2.8 Gene ontology analysis

Genes in this analysis were initially annotated using Ensembl three-spine stickleback gene stable IDs. To conduct ontology analysis I first converted the Ensembl Stickleback gene stable IDs to Ensembl Zebrafish gene stable IDs using *biomaRt* (Durinck et al., 2009; Kinsella et al., 2011). The Metascape package (Zhou et al., 2019) was used on this converted data to interpret the list of DE genes produced from each analysis (minimum overlap 3, p value cut-off 0.01 and minimum enrichment 1.5). Gene ontology (GO) enrichment analysis relies on an accurate assessment of whether a particular gene ontology is overrepresented in a dataset. Therefore, in order to avoid sampling bias, background gene sets of only the expressed genes (non-zero total read), rather than the whole zebrafish genome, were used for each tissue (Timmons et al., 2015). The recommended maximum for Metascape gene ontology analysis is 3000 genes. Gene lists that exceeded this number for gene ontology analysis using Metascape were filtered to remove genes with an absolute LFC lower than 0.5.

As I considered gene modules across three groups, resulting in a large number of modules overall, gene ontology analysis was performed only on modules found to be significantly divergent, disrupted or plastic. The interpretation of module gene ontology was further simplified by selecting the top-level gene ontology terms containing the most significant lower-level GO term (based on adjusted p values (log q)).

3.3 Results

3.3.1 Gene expression data

Samples were sequenced with an average of ~26M read pairs and an average read length of 143 base pairs per sample. Reads aligned at an average rate of ~92% across all samples (minimum 88%, maximum 97%), with an average 57M aligned rears per sample.

Levene's testing showed that a small number of genes differed in expression variance between geothermal and ambient sticklebacks and between temperatures (Table 3-1). However, genes differed in variance between tissues, with 7197 out of 9642 genes expressed in both brain and liver tissue differing in variation, 6381 of which were more variable in the liver than brain. After normalization and low expression filtration, 13452 genes in the brain (13454 in *EdgeR* and 13452 in *LIMMA*) and 10506 genes in the liver (10744 in *EdgeR* and 10506 in *LIMMA*) were used for differential expression analysis. Pearson's product-moment correlation coefficients were found to be >0.8 for all differential expression analyses performed, indicating a strong positive correlation between the results of the two analysis methods. As *LIMMA* and *EdgeR* analyses were found to correlate, gene lists from both analysis methods were merged to select only DE genes found by both.

3.3.2 Divergence between geothermal and ambient sticklebacks

In relation to prediction 1, I tested for differential expression between geothermal and ambient sticklebacks, first at 12°C and then at 18°C in order to assess for DE that was robust to temperature. I found some robust divergence in the brain, with 24 genes divergent between geothermal and ambient

sticklebacks at both rearing temperatures (

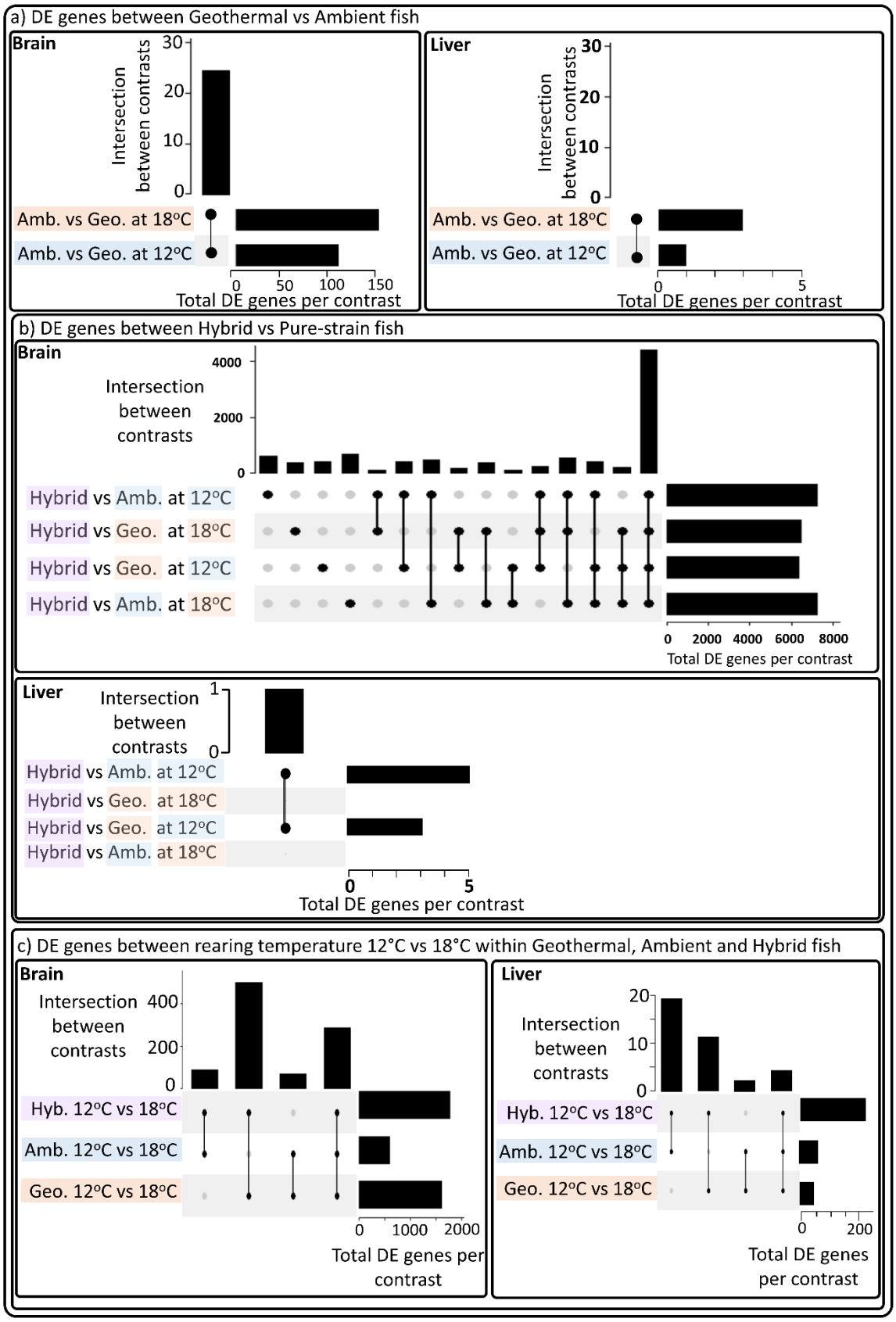


Figure 3-3a, Table 3-2). Geothermal and ambient fish were more divergent at 18˚C than at 12˚C, with a larger number of divergent genes and a broader

range of affected gene ontologies (

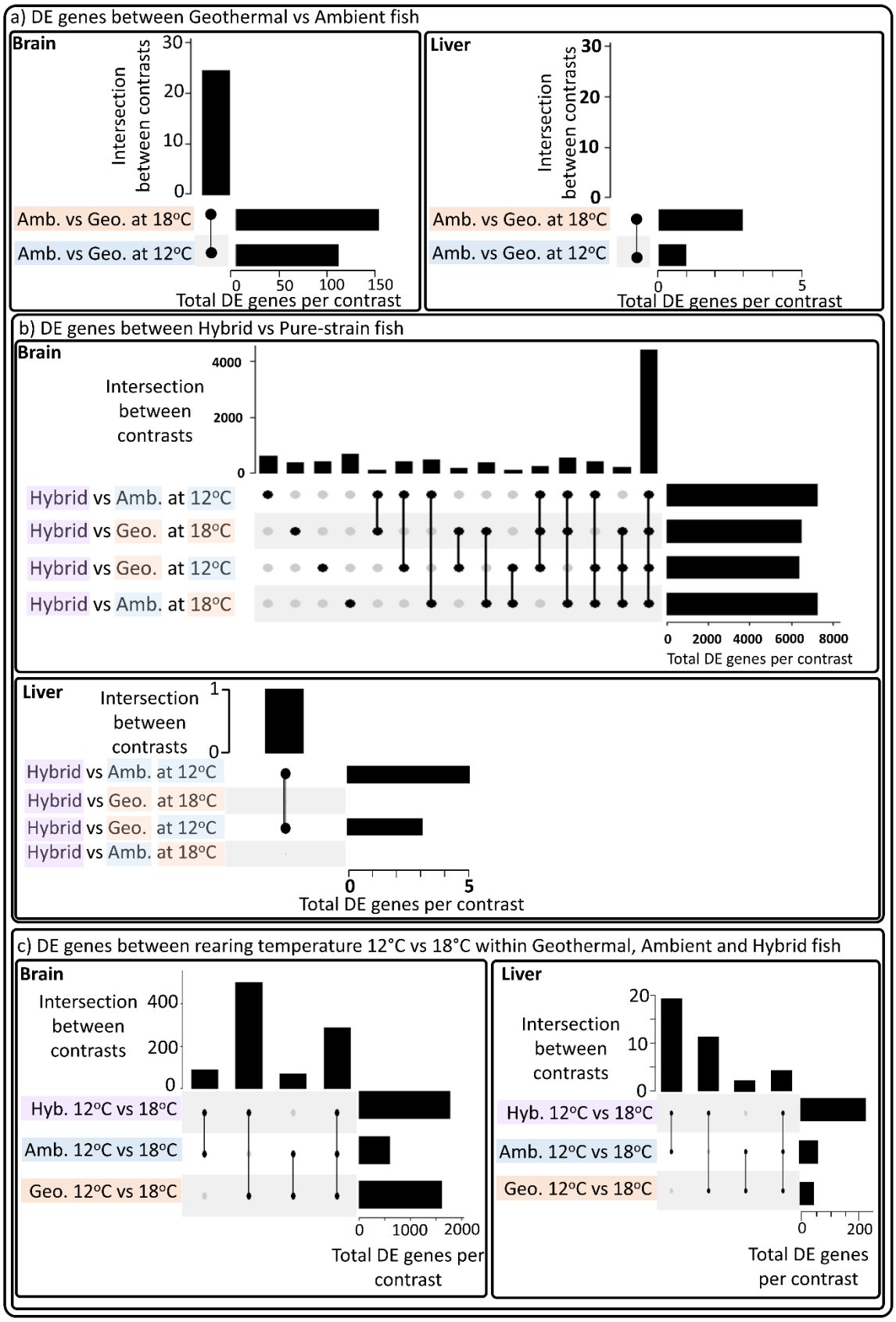


Figure 3-3a, and Table 3-2a). Divergence at both rearing temperatures included genes relating to the metabolic processes term (GO:0008152: metabolic process). Metabolic divergence depended on temperature, with divergence in

RNA splicing (GO:0008380) at 12^{o}C and divergence in monocarboxylic acid metabolic processes (GO:0032787) at 18^{o}C (Table 3-3).

Table 3-1 Numbers of genes that differ in variance, calculated using Levene's test on a gene-by-gene basis. Variance across samples within a given group calculated for each gene and p values adjusted using BH method (alpha = 0.05).

Contrast		Total genes	Number of genes differing in variance	
Liver versus brain tissue		9642	7197	6381 more variable in liver 826 more variable in brain
Ambient fish versus Geothermal fish	Brain	13244	0	-
	Liver	9642	1	1 more variable in geothermal fish
Ambient fish vs Hybrid fish	Brain	13244	15	11 more variable in hybrid fish
	Liver	9642	0	4 more variable in ambient fish
Geothermal fish vs Hybrid fish	Brain	13244	1	1 more variable in hybrid fish
	Liver	9642	0	-
Ambient fish at 12°C vs 18°C	Brain	13244	0	-
	Liver	9642	0	-
Geothermal fish at 12°C vs 18°C	Brain	13244	1	1 more variable at 12°C
	Liver	9642	1	1 more variable at 18°C
Hybrid fish at 12°C vs 18°C	Brain	13244	2	1 more variable at 12°C 1 more variable at 18°C
	Liver	9642	2	2 more variable at 12°C

Table 3-2 Numbers of differentially expressed genes found in pairwise contrasts, with EdgeR and LIMMA results included.

		Brain (*EdgeR: 13454/LIMMA: 13452)*			**Liver** (*EdgeR: 10744/LIMMA: 10506)*		
Analysis	**Comparison *(EdgeR/Limma)***	**Total DE genes**	**Direction**		**Total DE genes**	**Direction**	
Geothermal vs Ambient fish DE	**Pairwise contrasts**	**558 (740/583)**	**Downregulated in geothermal fish**	**Upregulated in geothermal fish**	**16 (70/17)**	**Downregulated in geothermal fish**	**Upregulated in geothermal fish**
	Ambient vs geothermal at 12	114 (*265/115)*	58 (*132/59)*	56 (*133/56)*	1 (*10/1*)	1 (*7/1*)	0 (*3/0*)
	Ambient vs geothermal at 18	148 (*182/201)*	79 (*87/109*)	69 *(95/92)*	4 (*68/4*)	1 (*52/1*)	3 (*16/3*)
Hybrid vs Straight Cross DE	**Pairwise contrasts**	**8403 (8879/8669)**	**Downregulated in hybrids**	**Upregulated in hybrids**	**18 (198/23)**	**Downregulated in hybrids**	**Upregulated in hybrids**
	Hybrid vs Ambient at 12˚C	6667 (7435/7225)	3425 (3654/3771)	3242 (3781/3454)	16 (25/18)	4 (8/4)	12 (17/14)
	Hybrid vs Geothermal at 18˚C	6023 (6569/6430)	3102 (3291/3368)	2921 (3278/3062)	0 (0/0)	0 (0/0)	0 (0/0)
	Hybrid vs Ambient at 18˚C	6986 (7284/7222)	3358 (3578/3686)	3297 (3706/3536)	0 (66/0)	0 (57/0)	0 (9/0)
	Hybrid vs Geothermal at 12˚C	6134 (6513/6349)	3142 (3290/3414)	2791 (3223/2935)	4 (12/5)	1 (7/1)	3 (5/4)
Geothermal vs Ambient plasticity	**Pairwise contrasts**	**1878 (2046/1981)**	**Downregulated at 18˚C**	**Upregulated at 18˚C**	**184(451/192)**	**Downregulated at 18˚C**	**Upregulated at 18˚C**
	Geothermal at 12˚C vs 18˚C	1526 (1653/1609)	819 (865/856)	707 (788/753)	44 (62/63)	28 (37/40)	16 (25/23)
	Ambient at 12˚C vs 18˚C	556 (640/599)	287 (323/315)	269 (317/284)	54 (311/56)	34 (71/36)	20 (240/20)
Hybrid plasticity	**Pairwise**	**2899 (3113/3025)**	**Downregulated at 18˚C**	**Upregulated at 18˚C**	**600 (650/1012)**	**Downregulated at 18˚C**	**Upregulated at 18˚C**
	Hybrid at 12˚C vs 18˚C	1670 (1822/1770)	736 (888/824)	814 (934/946)	219 (348/259)	127 (155/160)	92 (193/99)

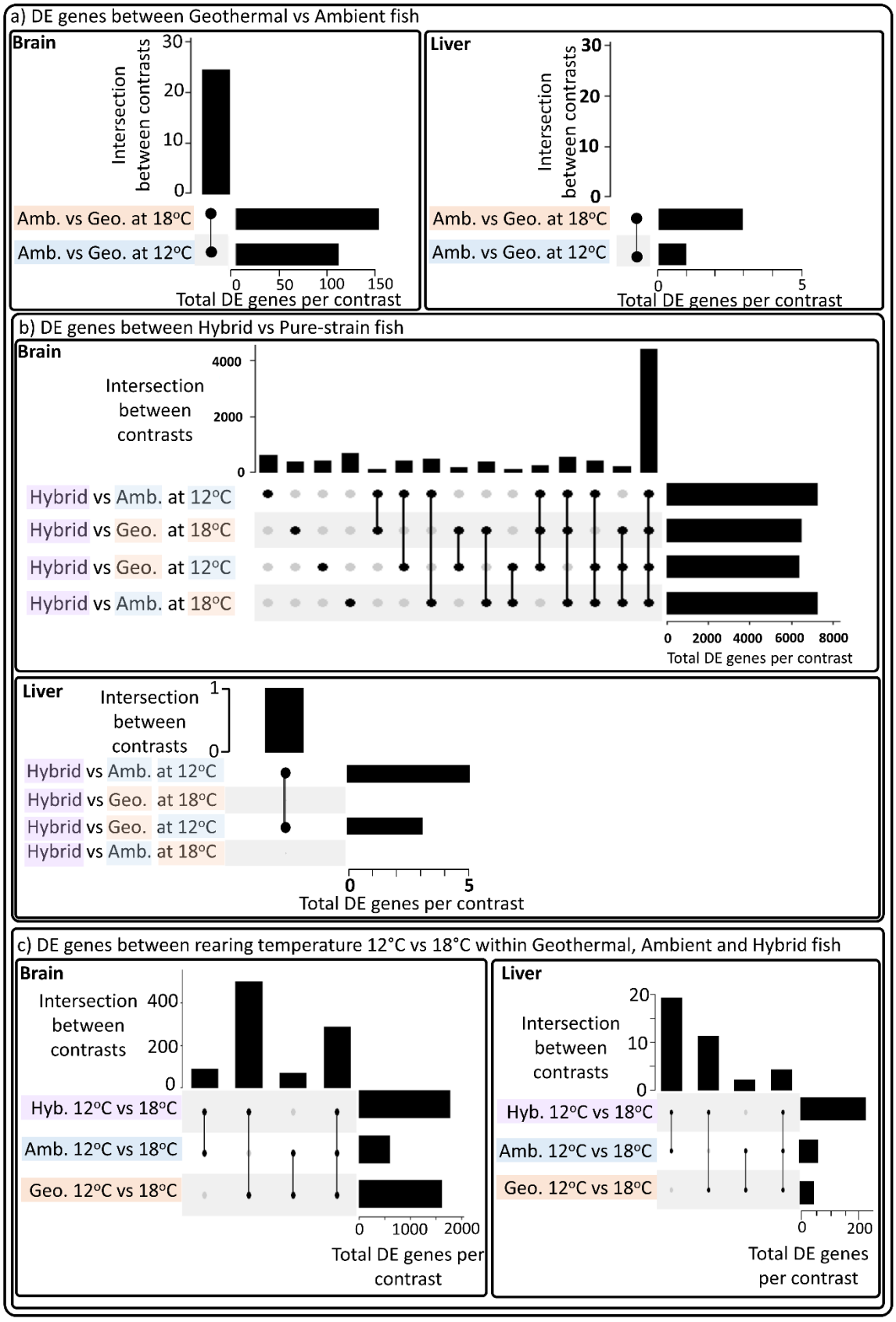
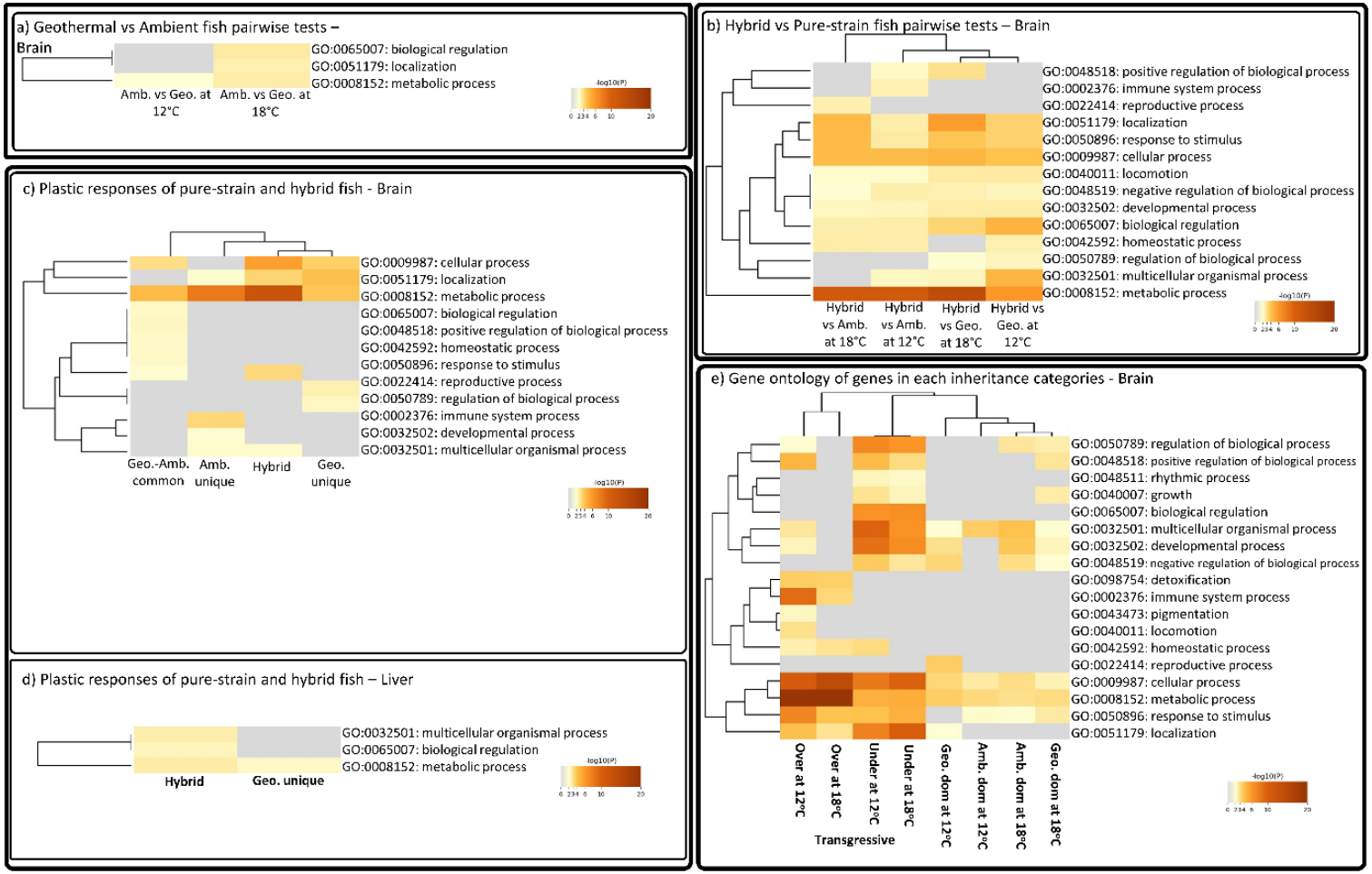


Figure 3-3 Upset plots (R package "ComplexHeatmaps") showing numbers of differentially expressed genes per pairwise contrast (horizontal bars) and the number of genes found to be differentially expressed in multiple contrasts (vertical bars)



(Previous page) Figure 3-4 Gene ontology terms found to be enriched in differentially expressed genes between experimental contrasts. a) geothermal versus ambient fish brain gene expression, b) hybrid versus pure-strain brain gene expression, c) plastic responses of pure-strain and hybrid fish in brain gene expression, d) plastic responses of pure-strain and hybrid fish in liver gene expression and e) gene ontology of genes in each inheritance category in the brain

Table 3-3 Gene ontology of genes DE between geothermal and ambient sticklebacks at 12°C or 18°C rearing temperatures.

		Parent GO	GO term	Log(q)	Log(P)	Z	# genes
Brain	**Ambient vs Geothermal at 12°C**	GO:0008152 metabolic process	RNA splicing (GO:0008380)	0	-2.0	3.3	5
	Ambient vs Geothermal at 18°C	GO:0008152 metabolic process	monocarboxylic acid metabolic process (GO:0032787)	0	-2.0	3.1	6
			olefinic compound metabolic process (GO:0120254)	0	-2.7	5.5	3
			hormone metabolic process (GO:0042445)	0	-2.4	4.6	3
		GO:0051179 localization	phospholipid transport (GO:0015914)	0	-2.6	5.1	3
			sodium ion transmembrane transport (GO:0035725)	0	-2.0	3.9	3
			organophosphate ester transport (GO:0015748)	0	-2.0	3.9	3
		GO:0065007 biological regulation	regulation of hormone levels (GO:0010817)	0	-2.7	4.8	4

3.3.2.1 Divergence in gene co-expression between geothermal and ambient sticklebacks

I assessed the preservation of gene co-expression modules between geothermal and ambient sticklebacks. I found some evidence that geothermal and ambient sticklebacks differed in gene co-expression. Brain gene expression clustered into 37 eigengene modules in geothermal fish and 34 in ambient fish (Table 3-4, Figure 3-5). Two geothermal brain modules were not preserved in ambient fish (Table 3-4), suggesting that the genes within these two geothermal brain modules do not co-express in the same way. These divergent modules were related to developmental (GO:0032502) and cellular (GO:0009987) processes, particularly those relating to muscle development and collagen organization (Table 3-9).

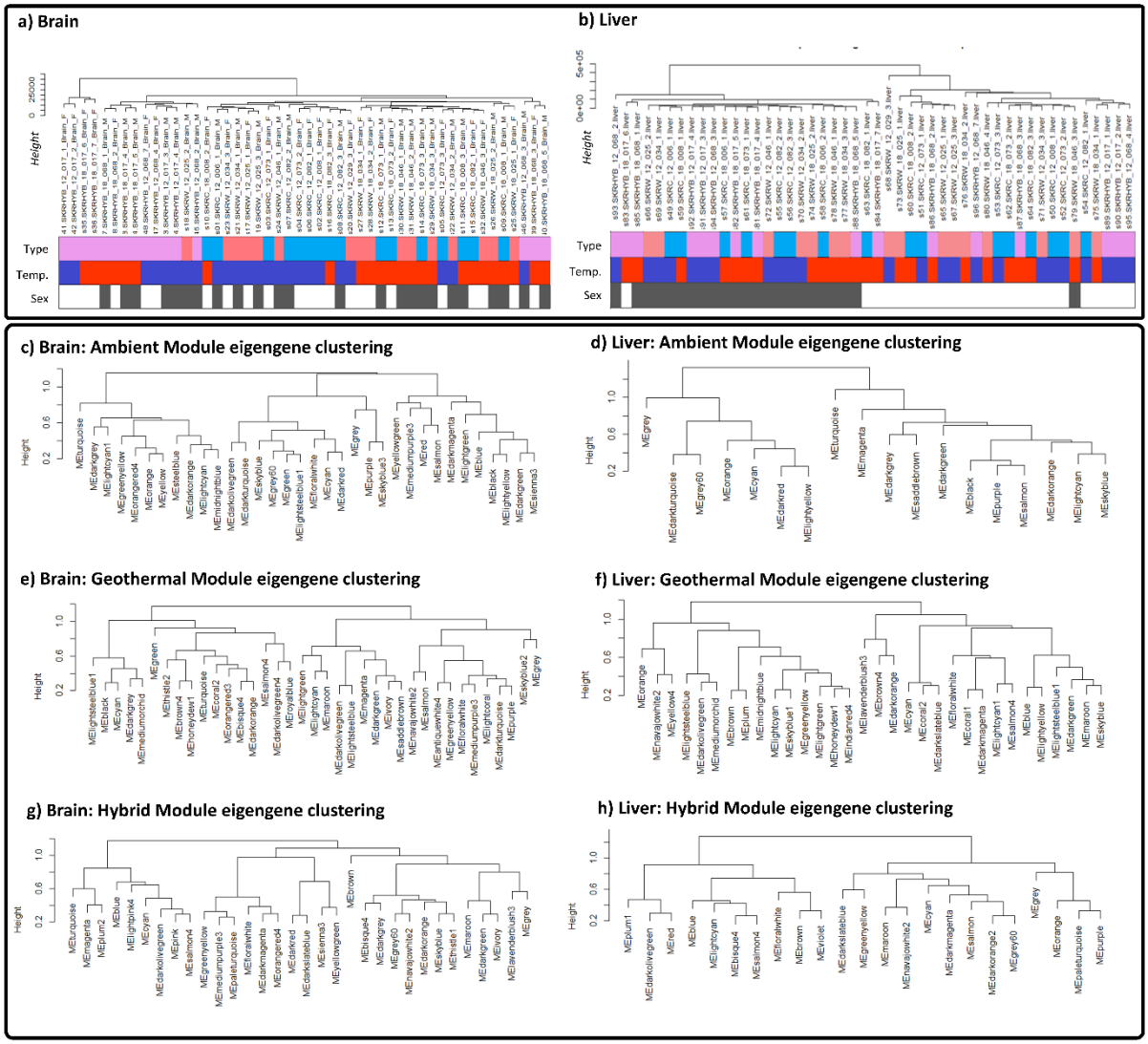


Figure 3-5 top panel: WGCNA clustering of samples in a) brain and b) liver data set, with fish type (magenta = hybrid, red = geothermal, blue= ambient), temperature (blue = 12˚C, red = 18˚C) and sex (white = female, black = male). Bottom panel: WGCNA Clustering of module eigengenes in brain gene expression data, in a) ambient fish, b) geothermal fish and c) hybrid fish. And of module eigengenes in liver gene expression data, in d) ambient fish, e) geothermal fish and f) hybrid fish

Table 3-4 Data concerning gene co-expression modules of the brains and livers of ambient, geothermal and hybrid fish. Module preservation was performed reciprocally between geothermal and ambient fish, as well as between hybrid fish and each pure-strain fish. The relationship between module co-expression and temperature was tested using Gaussian GLMs. For each module found to be significant in either preservation or glm analysis, the most significantly enriched parent GO term is reported. Grey modules represent genes that did not cluster into a module, and gold modules represent a random sample of 1000 genes.

			Module preservation		Plasticity glm: Temperature				Plasticity glm: Intercept					
Dataset	Module	# genes	Med. Rank pres (qual)	Z sum. pres (qual)	Est.	Std. Error	t	P	Est.	Std. Error	t	P	Res. Dev.	Most Sig GO term
Ambient Brain	AB_black	660	23 (15)	17 (79)	0.13	0.13	1.03	0.3230	-0.07	0.09	-0.72	0.4810	0.93	
	AB_blue	2000	21 (32.5)	26 (55)	0.07	0.13	0.56	0.5870	-0.04	0.09	-0.39	0.7000	0.98	
	AB_cyan	512	20 (19.5)	18 (61)	0.09	0.13	0.68	0.5090	-0.04	0.09	-0.48	0.6390	0.97	
	AB_darkgreen	202	28 (15)	6.3 (39)	0.09	0.13	0.72	0.4870	-0.05	0.09	-0.51	0.6210	0.96	
	AB_darkgrey	190	33 (11.5)	3.5 (41)	-0.16	0.13	-1.26	0.2300	0.08	0.09	0.89	0.3900	0.90	
	AB_darkmagenta	133	27 (30.5)	5.2 (23)	-0.22	0.12	-1.85	0.0849	0.11	0.08	1.31	0.2109	0.80	
	AB_darkolivegreen	134	2 (2.5)	16 (43)	0.09	0.13	0.66	0.5190	-0.04	0.09	-0.47	0.6400	0.97	
	AB_darkorange	179	16 (18)	12 (39)	-0.25	0.12	-2.16	**0.0489**	0.12	0.08	1.53	0.1496	0.75	GO:0051179 localization
	AB_darkred	522	13 (22)	24 (61)	0.36	0.09	3.83	**0.0019**	-0.18	0.07	-2.71	0.0170	0.49	GO:0002376 immune system process
	AB_darkturquoise	334	13 (27)	17 (45)	0.23	0.12	1.91	0.0774	-0.11	0.08	-1.35	0.1992	0.79	
	AB_floralwhite	71	6 (5)	9.5 (29)	0.28	0.11	2.59	**0.0212**	-0.14	0.08	-1.84	0.0879	0.68	none
	AB_green	800	5 (21)	41 (78)	0.43	0.07	6.36	**0.0001**	-0.22	0.05	-4.50	0.0005	0.26	GO:0008152 metabolic process
	AB_greenyellow	1320	9 (32)	39 (52)	-0.40	0.08	-5.14	**0.0002**	0.20	0.06	3.64	0.0027	0.35	GO:0008152 metabolic process
	AB_grey60	402	22 (24.5)	16 (49)	0.23	0.12	1.94	0.0725	-0.12	0.08	-1.37	0.1912	0.79	
	AB_lightcyan	386	20 (11)	15 (61)	-0.32	0.10	-3.14	**0.0073**	0.16	0.07	2.22	0.0435	0.59	GO:0009987 cellular process
	AB_lightcyan1	80	33 (9.5)	3.3 (27)	-0.16	0.13	-1.27	0.2250	0.08	0.09	0.90	0.3840	0.90	
	AB_lightgreen	237	29 (20)	7.5 (42)	-0.05	0.13	-0.36	0.7230	0.02	0.09	0.26	0.8020	0.99	
	AB_lightsteelblue1	91	4 (1)	13 (33)	0.40	0.08	5.06	**0.0002**	-0.20	0.06	-3.58	0.0030	0.35	GO:0008152 metabolic process
	AB_lightyellow	221	27 (26)	7.3 (34)	-0.06	0.13	-0.44	0.6700	0.03	0.09	0.31	0.7620	0.99	
	AB_mediumpurple3	102	14 (23)	8.7 (26)	0.11	0.13	0.88	0.3960	-0.06	0.09	-0.62	0.5460	0.95	
	AB_midnightblue	273	7 (17)	19 (48)	-0.35	0.09	-3.75	**0.0022**	0.18	0.07	2.65	0.0190	0.50	GO:0008152 metabolic process
	AB_orange	182	3 (3)	16 (52)	-0.40	0.08	-4.88	**0.0002**	0.20	0.06	3.45	0.0039	0.37	GO:0048519 -ve reg. of biological process
	AB_orangered4	103	1 (3.5)	15 (38)	-0.19	0.12	-1.52	0.1520	0.09	0.09	1.07	0.3020	0.86	
	AB_purple	336	8 (13)	26 (48)	0.21	0.12	1.75	0.1020	-0.11	0.09	-1.24	0.2360	0.82	
	AB_red	625	32 (28.5)	7.3 (52)	-0.10	0.13	-0.79	0.4450	0.05	0.09	0.56	0.5870	0.96	
	AB_salmon	310	14 (28.5)	16 (36)	-0.04	0.13	-0.32	0.7510	0.02	0.09	0.23	0.8220	0.99	
	AB_sienna3	128	31 (7)	3.9 (35)	-0.12	0.13	-0.96	0.3540	0.06	0.09	0.68	0.5080	0.94	
	AB_skyblue	172	12 (25)	13 (30)	0.40	0.08	4.83	**0.0003**	-0.20	0.06	-3.42	0.0042	0.37	GO:0009987 cellular process
	AB_skyblue3	117	21 (9.5)	9.2 (35)	0.15	0.13	1.15	0.2680	-0.07	0.09	-0.82	0.4280	0.91	
	AB_steelblue	154	24 (8)	8.8 (40)	-0.15	0.13	-1.16	0.2670	0.07	0.09	0.82	0.4270	0.91	
	AB_turquoise	1682	12 (30.5)	49 (60)	-0.13	0.13	-0.97	0.3500	0.06	0.09	0.68	0.5050	0.94	
	AB_yellow	650	10 (6)	29 (94)	-0.17	0.13	-1.33	0.2050	0.08	0.09	0.94	0.3630	0.89	
	AB_yellowgreen	122	12 (30.5)	49 (60)	-0.01	0.13	-0.05	0.9610	0.00	0.09	0.04	0.9720	1.00	
	AB_grey	*23*	*32 (34)*	*1.1 (0.98)*										
	AB_gold		*29 (35)*	*10 (0.52)*										
Geothermal Brain	GB_antiquewhite4	289	20 (21)	15 (41)	0.01	0.13	0.08	0.9360	-0.01	0.09	-0.06	0.9550	1.00	
	GB_bisque4	1003	29 (35)	24 (65)	0.42	0.07	5.93	**0.0001**	-0.21	0.05	-4.20	0.0009	0.28	GO:0009987 cellular process
	GB_black	717	32 (21.5)	13 (60)	-0.10	0.13	-0.73	0.4760	0.05	0.09	0.52	0.6120	0.96	
	GB_brown4	98	10 (5.5)	11 (34)	0.33	0.10	3.32	**0.0051**	-0.17	0.07	-2.35	0.0341	0.56	GO:0009987 cellular process
	GB_coral2	74	3 (10)	16 (27)	0.03	0.13	0.20	0.8450	-0.01	0.09	-0.14	0.8900	1.00	
	GB_cyan	478	34 (30)	9.1 (42)	-0.17	0.13	-1.37	0.1930	0.09	0.09	0.97	0.3490	0.88	
	GB_darkgreen	1211	16 (33)	36 (63)	0.44	0.06	7.15	**0.0001**	0.44	0.06	7.15	0.0000	0.21	GO:0048519 -ve reg. of biological process
	GB_darkgrey	1504	21 (32)	31 (58)	-0.36	0.09	-3.85	**0.0018**	0.18	0.07	2.72	0.0165	0.49	GO:0008152 metabolic process
	GB_darkolivegreen	120	1 (7)	18 (35)	0.18	0.13	1.40	0.1820	-0.09	0.09	-0.99	0.3380	0.88	

	GB_darkolivegreen4	51	7 (5.5)	11 (24)	-0.01	0.13	-0.10	0.9210	0.01	0.09	0.07	0.9440	1.00	
	GB_darkorange	155	13 (2)	13 (43)	0.28	0.11	2.50	**0.0257**	-0.14	0.08	-1.77	0.0994	0.69	GO:0032501 multicellular organismal process
	GB_darkturquoise	735	24 (29)	23 (58)	-0.25	0.12	-2.13	0.0513	0.12	0.08	1.51	0.1541	0.76	
	GB_floralwhite	307	12 (15)	20 (49)	-0.39	0.08	-4.57	**0.0004**	0.19	0.06	3.23	0.0061	0.40	GO:0008152 metabolic process
	GB_green	502	27 (36)	17 (37)	0.11	0.13	0.86	0.4050	-0.06	0.09	-0.61	0.5530	0.95	
	GB_greenyellow	307	15 (23.5)	16 (41)	0.03	0.13	0.23	0.8250	-0.02	0.09	-0.16	0.8760	1.00	
	GB_honeydew1	195	23 (17)	14 (37)	0.18	0.12	1.47	0.1630	-0.09	0.09	-1.04	0.3160	0.87	
	GB_ivory	104	23 (11)	8.3 (30)	0.32	0.10	3.08	**0.0082**	-0.16	0.07	-2.18	0.0473	0.60	GO:0008152 metabolic process
	GB_lightcoral	171	30 (18)	8.4 (34)	-0.23	0.12	-1.93	0.0747	0.11	0.08	1.36	0.1949	0.79	
	GB_lightcyan	205	16 (34)	16 (28)	0.07	0.13	0.53	0.6070	-0.03	0.09	-0.37	0.7150	0.98	
	GB_lightgreen	192	19 (21)	13 (31)	0.22	0.12	1.88	0.0809	-0.11	0.08	-1.33	0.2046	0.80	
	GB_lightsteelblue	59	6 (1)	8.9 (27)	-0.02	0.13	-0.12	0.9090	0.01	0.09	0.08	0.9360	1.00	
	GB_lightsteelblue1	199	10 (19)	19 (33)	-0.05	0.13	-0.39	0.6990	0.03	0.09	0.28	0.7840	0.99	
	GB_magenta	326	37 (24.5)	**0.65 (37)**	0.13	0.13	1.01	0.3300	-0.07	0.09	-0.71	0.4870	0.93	GO:0032502 developmental process
	GB_maroon	341	4 (27.5)	35 (40)	-0.05	0.13	-0.40	0.6970	0.03	0.09	0.28	0.7830	0.99	
	GB_mediumorchid	73	22 (3)	7.4 (29)	-0.05	0.13	-0.34	0.7370	0.02	0.09	0.24	0.8120	0.99	
	GB_mediumpurple3	695	12 (31)	29 (49)	-0.43	0.07	-6.47	**<0.0001**	0.22	0.05	4.57	0.0004	0.25	GO:0051179 localization
	GB_navajowhite2	83	29 (8)	8.2 (30)	-0.22	0.12	-1.85	0.0859	0.11	0.08	1.31	0.2125	0.80	
	GB_orangered3	63	26 (9)	5.9 (26)	0.20	0.12	1.63	0.1250	-0.10	0.09	-1.15	0.2680	0.84	
	GB_purple	613	17 (25)	27 (56)	-0.22	0.12	-1.84	0.0874	0.11	0.08	1.30	0.2148	0.81	
	GB_royalblue	178	30 (24)	8.2 (30)	0.06	0.13	0.44	0.6670	-0.03	0.09	-0.31	0.7600	0.99	
	GB_saddlebrown	297	16 (14)	17 (46)	0.17	0.13	1.33	0.2060	-0.08	0.09	-0.94	0.3650	0.89	
	GB_salmon	511	5 (27)	42 (49)	-0.23	0.12	-1.95	0.0714	0.12	0.08	1.38	0.1894	0.79	
	GB_salmon4	87	37 (12.5)	**1.4 (25)**	-0.16	0.13	-1.24	0.2350	0.08	0.09	0.88	0.3950	0.90	GO:0009987 cellular process
	GB_skyblue2	69	33 (16)	4.5 (20)	-0.15	0.13	-1.20	0.2520	0.08	0.09	0.85	0.4120	0.91	
	GB_thistle2	88	5 (4)	16 (31)	0.29	0.11	2.62	**0.0203**	-0.14	0.08	-1.85	0.0854	0.67	GO:0032501 multicellular organismal process
	GB_turquoise	1329	6 (12.5)	77 (88)	0.05	0.13	0.38	0.7080	-0.03	0.09	-0.27	0.7910	0.99	
	GB_grey	*24*	*37 (37.5)*	*-0.37 (-0.22)*										
	GB_gold		*33 (37.5)*	*11 (-0.41)*										
Ambient liver	AL_black	431	6 (8)	22 (29)	-0.14	0.13	-1.08	0.2980	0.07	0.09	0.76	0.4580	0.92	
	AL_cyan	1335	13 (14)	29 (39)	0.09	0.13	0.71	0.4880	-0.05	0.09	-0.50	0.6220	0.97	
	AL_darkgreen	177	8 (12.5)	15 (15)	-0.07	0.13	-0.50	0.6240	0.03	0.09	0.35	0.7290	0.98	
	AL_darkgrey	139	16 (11)	6.6 (17)	-0.13	0.13	-0.98	0.3450	0.06	0.09	0.69	0.5010	0.94	GO:0051179 localization
	AL_darkorange	130	11 (6)	7.7 (18)	0.13	0.13	1.00	0.3350	-0.06	0.09	-0.71	0.4920	0.93	GO:0032502 developmental process
	AL_darkred	3056	6 (17)	35 (32)	-0.13	0.13	-1.05	0.3130	0.07	0.09	0.74	0.4710	0.93	
	AL_darkturquoise	158	4 (7)	16 (22)	-0.03	0.13	-0.26	0.7990	0.02	0.09	0.18	0.8570	1.00	
	AL_grey60	214	12 (12.5)	11 (19)	-0.18	0.12	-1.46	0.1670	0.09	0.09	1.03	0.3200	0.87	
	AL_lightcyan	1225	10 (10.5)	23 (48)	0.24	0.12	2.00	0.0651	-0.12	0.08	-1.42	0.1788	0.78	
	AL_lightyellow	190	14 (9)	7.1 (19)	-0.23	0.12	-1.96	0.0697	0.12	0.08	1.39	0.1866	0.78	
	AL_magenta	370	17 (15.5)	**0.63 (21)**	0.10	0.13	0.74	0.4730	-0.05	0.09	-0.52	0.6100	0.96	GO:0050896 response to stimulus
	AL_orange	138	1 (4)	23 (18)	0.00	0.13	-0.03	0.9760	0.00	0.09	0.02	0.9830	1.00	
	AL_purple	350	5 (1)	21 (35)	0.02	0.13	0.13	0.8950	-0.01	0.09	-0.10	0.9260	1.00	
	AL_saddlebrown	51	2 (3)	9 (13)	-0.08	0.13	-0.64	0.5340	0.04	0.09	0.45	0.6590	0.97	
	AL_salmon	284	10 (5)	15 (27)	-0.08	0.13	-0.64	0.5360	0.04	0.09	0.45	0.6600	0.97	
	AL_skyblue	99	9 (2)	7 (16)	0.14	0.13	1.08	0.3000	-0.07	0.09	-0.76	0.4590	0.92	
	AL_turquoise	2240	18 (15.5)	6.4 (36)	0.16	0.13	1.28	0.2230	-0.08	0.09	-0.90	0.3830	0.90	
	AL_grey	*157*	*19 (19)*	*0.93 (-5)*										
	AL_gold		*12 (18)*	*28 (1.1)*										
Geothermal liver	GL_blue	2150	26 (22.5)	21 (61)	-0.04	0.13	-0.33	0.7470	0.02	0.09	0.23	0.8190	0.99	
	GL_brown	518	20 (16.5)	21 (41)	-0.08	0.13	-0.57	0.5780	0.04	0.09	0.40	0.6930	0.98	
	GL_brown4	90	30 (23.5)	2.1 (15)	0.22	0.12	1.87	0.0832	-0.11	0.08	-1.32	0.2082	0.80	
	GL_coral1	64	29 (9)	**1.7 (17)**	-0.35	0.10	-3.67	**0.0026**	0.17	0.07	2.59	0.0213	0.51	none
	GL_coral2	61	21 (6)	5.3 (18)	-0.14	0.13	-1.07	0.3030	0.07	0.09	0.76	0.4620	0.92	
	GL_cyan	510	5 (28)	30 (37)	-0.07	0.13	-0.51	0.6220	0.03	0.09	0.36	0.7270	0.98	
	GL_darkgreen	352	5 (15.5)	26 (36)	-0.23	0.12	-1.92	0.0754	0.11	0.08	1.36	0.1959	0.79	
	GL_darkmagenta	106	33 (10)	**-0.87 (21)**	-0.35	0.10	-3.61	**0.0028**	0.17	0.07	2.56	0.0229	0.52	GO:0050896 response to stimulus
	GL_darkolivegreen	391	19 (22.5)	17 (31)	-0.07	0.13	-0.53	0.6080	0.03	0.09	0.37	0.7160	0.98	
	GL_darkorange	124	17 (7)	7 (24)	0.17	0.13	1.32	0.2080	-0.08	0.09	-0.93	0.3660	0.89	
	GL_darkslateblue	581	32 (17)	**1.3 (45)**	-0.17	0.13	-1.37	0.1940	0.09	0.09	0.97	0.3510	0.88	GO:0048519 -ve reg. of biological process
	GL_floralwhite	92	26 (25.5)	7.8 (15)	-0.06	0.13	-0.46	0.6520	0.03	0.09	0.33	0.7490	0.99	
	GL_greenyellow	421	12 (28.5)	22 (31)	-0.10	0.13	-0.73	0.4790	0.05	0.09	0.51	0.6150	0.96	
	GL_honeydew1	1226	8 (32)	38 (40)	0.18	0.13	1.41	0.1800	-0.09	0.09	-1.00	0.3350	0.88	
	GL_indianred4	1078	8 (30)	37 (48)	-0.13	0.13	-1.02	0.3270	0.07	0.09	0.72	0.4840	0.93	
	GL_lavenderblush3	152	24 (21)	6.5 (20)	0.22	0.12	1.80	0.0927	-0.11	0.09	-1.28	0.2228	3.70	
	GL_lightcyan	186	9 (4)	14 (35)	0.12	0.13	0.89	0.3900	-0.06	0.09	-0.63	0.5410	0.95	
	GL_lightcyan1	94	13 (16.5)	10 (19)	0.08	0.13	0.58	0.5730	-0.04	0.09	-0.41	0.6890	0.98	
	GL_lightgreen	156	15 (5)	9.6 (28)	-0.07	0.13	-0.51	0.6160	0.03	0.09	0.36	0.7220	0.98	

	GL_lightsteelblue	40		10 (2)	6.1 (16)	-0.26	0.11	-2.31	**0.0369**	0.13	0.08	1.63	0.1253	0.72	GO:0009987 cellular process
	GL_lightsteelblue1	173		20 (18.5)	8.9 (22)	0.14	0.13	1.09	0.2950	-0.07	0.09	-0.77	0.4540	0.92	
	GL_lightyellow	415		28 (27)	7.8 (33)	-0.21	0.12	-1.77	0.0986	0.11	0.09	1.25	0.2314	0.82	
	GL_maroon	565		3 (31)	33 (37)	-0.04	0.13	-0.27	0.7930	0.02	0.09	0.19	0.8520	0.99	
	GL_mediumorchid	160		25 (13.5)	7 (24)	-0.33	0.10	-3.27	**0.0056**	0.16	0.07	2.31	0.0366	0.57	GO:0008152 metabolic process
	GL_midnightblue	189		16 (25.5)	12 (20)	0.23	0.12	1.89	0.0797	-0.11	0.08	-1.34	0.2028	0.80	
	GL_navajowhite2	74		2 (8)	18 (18)	-0.21	0.12	-1.77	0.0986	0.11	0.09	1.25	0.2314	0.82	
	GL_orange	210		11 (20)	15 (23)	0.08	0.13	0.63	0.5380	-0.04	0.09	-0.45	0.6620	0.97	
	GL_plum	50		18 (1)	6.1 (21)	-0.19	0.12	-1.57	0.1380	0.10	0.09	1.11	0.2850	0.85	
	GL_salmon4	77		21 (12)	5.6 (16)	-0.18	0.12	-1.49	0.1590	0.09	0.09	1.05	0.3110	0.86	
	GL_skyblue	328		1 (13.5)	33 (34)	0.03	0.13	0.23	0.8210	-0.02	0.09	-0.16	0.8730	1.00	
	GL_skyblue1	55		12 (3)	7.3 (20)	0.21	0.12	1.72	0.1070	-0.10	0.09	-1.22	0.2430	0.83	
	GL_yellow4	56		30 (11)	**1.6 (14)**	0.16	0.13	1.25	0.2310	-0.08	0.09	-0.89	0.3910	0.90	GO:0050789 reg. of biological process
	GL_gold			*21 (33)*											
Hybrid brain	HB_bisque4	100	vs Amb. vs Geo.	24 (9) 32 (9)	6.2 (32) 4.7 (32)	-0.14	0.13	-1.07	0.3010	0.07	0.09	0.76	0.4610	0.92	
	HB_blue	1798	vs Amb. vs Geo.	13 (31) 17 (31)	33 (64) 40 (64)	0.39	0.08	4.66	**0.0004**	-0.19	0.06	-3.30	0.0053	0.39	GO:0008152 metabolic process
	HB_brown	705	vs Amb. vs Geo.	27 (21.5) 26 (21.5)	9.8 (76) 21 (76)	-0.13	0.13	-0.98	0.3430	0.06	0.09	0.70	0.4990	0.94	
	HB_cyan	393	vs Amb. vs Geo.	9 (18) 16 (18)	26 (52) 20 (52)	0.03	0.13	0.22	0.8270	-0.01	0.09	-0.16	0.8770	1.00	
	HB_darkgreen	1049	vs Amb. vs Geo.	14 (29) 3 (29)	32 (79) 49 (79)	-0.44	0.06	-7.07	**0.0001**	0.22	0.04	5.00	0.0002	0.22	GO:0008152 metabolic process
	HB_darkgrey	188	vs Amb. vs Geo.	31 (11.5) 27 (11.5)	5.6 (43) 21 (43)	-0.24	0.12	-2.07	0.0580	0.12	0.08	1.46	0.1660	0.77	
	HB_darkmagenta	165	vs Amb. vs Geo.	22 (5) 15 (5)	7.8 (46) 12 (46)	-0.01	0.13	-0.08	0.9390	0.01	0.09	0.06	0.9570	1.00	
	HB_darkolivegreen	458	vs Amb. vs Geo.	29 (28) 31 (28)	10 (53) 5 (53)	-0.10	0.13	-0.79	0.4440	0.05	0.09	0.56	0.5860	0.96	
	HB_darkorange	752	vs Amb. vs Geo.	16 (26) 20 (26)	27 (63) 28 (63)	-0.24	0.12	-2.06	0.0587	0.12	0.08	1.46	0.1676	0.77	
	HB_darkred	493	vs Amb. vs Geo.	4 (25) 13 (25)	47 (56) 28 (56)	0.08	0.13	0.60	0.5590	-0.04	0.09	-0.42	0.6780	0.97	
	HB_darkslateblue	97	vs Amb. vs Geo.	2 (7) 1 (7)	20 (36) 17 (36)	-0.18	0.12	-1.44	0.1720	0.09	0.09	1.02	0.3260	0.87	
	HB_floralwhite	616	vs Amb. vs Geo.	11 (22.5) 20 (22.5)	25 (66) 26 (66)	0.34	0.10	3.42	**0.0042**	-0.17	0.07	-2.42	0.0298	0.54	GO:0009987 cellular process
	HB_greenyellow	441	vs Amb. vs Geo.	21 (16) 23 (16)	14 (65) 18 (65)	-0.15	0.13	-1.21	0.2470	0.08	0.09	0.85	0.4070	0.91	
	HB_grey60	244	vs Amb. vs Geo.	7 (6.5) 24 (6.5)	24 (53) 13 (53)	-0.17	0.13	-1.32	0.2070	0.08	0.09	0.94	0.3650	0.89	
	HB_ivory	430	vs Amb. vs Geo.	26 (17) 7 (17)	11 (64) 27 (64)	-0.17	0.13	-1.40	0.1840	0.09	0.09	0.99	0.3400	0.88	
	HB_lavenderblush3	47	vs Amb. vs Geo.	24 (19) 28 (19)	5.2 (18) 4.9 (18)	-0.23	0.12	-1.94	0.0730	0.12	0.08	1.37	0.1920	0.79	
	HB_lightpink4	63	vs Amb. vs Geo.	5 (2) 9 (2)	15 (31) 11 (31)	0.19	0.12	1.53	0.1480	-0.09	0.09	-1.08	0.2980	0.86	
	HB_magenta	303	vs Amb. vs Geo.	21 (20) 16 (20)	13 (47) 18 (47)	0.22	0.12	1.85	0.0863	-0.11	0.08	-1.30	0.2131	0.80	
	HB_maroon	69	vs Amb. vs Geo.	28 (8) 11 (8)	3.4 (27) 8.7 (27)	-0.15	0.13	-1.16	0.2660	0.07	0.09	0.82	0.4260	0.91	
	HB_mediumpurple3	148	vs Amb. vs Geo.	10 (11.5) 17 (11.5)	16 (37) 13 (37)	0.04	0.13	0.33	0.7460	-0.02	0.09	-0.23	0.8180	0.99	
	HB_navajowhite2	79	vs Amb. vs Geo.	18 (14) 30 (14)	8.4 (28) 5.6 (28)	-0.25	0.12	-2.12	0.0521	0.12	0.08	1.50	0.1555	0.76	
	HB_orangered4	439	vs Amb. vs Geo.	19 (21.5) 13 (21.5)	17 (59) 22 (59)	-0.01	0.09	-0.12	0.9090	0.02	0.13	0.16	0.8720	1.00	
	HB_paleturquoise	177	vs Amb. vs Geo.	2 (9.5) 5 (9.5)	36 (46) 17 (46)	0.00	0.13	0.01	0.9960	0.00	0.09	0.00	0.9970	1.00	
	HB_pink	1512	vs Amb. vs Geo.	23 (30) 22 (30)	22 (71) 32 (71)	0.28	0.11	2.56	**0.0226**	-0.14	0.08	-1.81	0.0916	0.68	GO:0051179 localization
	HB_plum2	94	vs Amb. vs Geo.	11 (15.5) 21 (15.5)	12 (31) 8.4 (31)	-0.14	0.13	-1.09	0.2940	0.07	0.09	0.77	0.4540	0.92	
	HB_salmon4	85	vs Amb. vs Geo.	16 (3) 11 (3)	8.7 (36) 11 (36)	0.15	0.13	1.14	0.2730	-0.07	0.09	-0.81	0.4330	0.91	
	HB_sienna3	677	vs Amb. vs Geo.	16 (25.5) 7 (25.5)	25 (69) 33 (69)	0.07	0.13	0.53	0.6050	-0.04	0.09	-0.38	0.7140	0.98	
	HB_skyblue	181	vs Amb. vs Geo.	3 (1) 5 (1)	19 (55) 17 (55)	-0.43	0.07	-6.43	**<0.0001**	0.22	0.05	4.55	0.0005	0.25	GO:0008152 metabolic process
	HB_thistle1	87	vs Amb. vs Geo.	6 (3.5) 10 (3.5)	13 (35) 11 (35)	-0.41	0.08	-5.49	**0.0001**	0.21	0.05	3.88	0.0017	0.32	GO:0051179 localization
	HB_turquoise	1253	vs Amb. vs Geo.	6 (25.5) 2 (25.5)	75 (82) 70 (82)	0.06	0.13	0.48	0.6420	-0.03	0.09	-0.34	0.7420	0.98	
	HB_yellowgreen	159	vs Amb. vs Geo.	31 (13.5) 21 (13.5)	0.69 (43) 13 (43)	0.17	0.13	1.35	0.2000	-0.08	0.09	-0.95	0.3580	0.89	

HB_grey	*151*	*vs Amb.*	*33 (33)*	*-0.56 (-3.3)*										
		vs Geo.	*33 (33)*	*-0.49 (-3.3)*										
HB_gold		*vs Amb.*	*28 (32)*	*12 (1.6)*										
		vs Geo.	*28 (32)*	*20 (1.6)*										
Hybrid liver														
HL_bisque4	719	vs Amb.	4 (16)	39 (51)	0.00	0.13	-0.03	0.9770	0.00	0.09	0.02	0.9830	7.04	
		vs Geo.	8 (16)	30 (51)										
HL_blue	529	vs Amb.	2 (11.5)	34 (45)	0.03	0.13	0.25	0.8040	-0.02	0.09	-0.18	0.8610	1.00	
		vs Geo.	3 (11.5)	38 (45)										
HL_brown	507	vs Amb.	5 (4)	31 (51)	0.11	0.13	0.86	0.4050	-0.06	0.09	-0.61	0.5540	0.95	
		vs Geo.	15 (4)	20 (51)										
HL_cyan	464	vs Amb.	10 (17.5)	22 (35)	0.11	0.13	0.81	0.4320	-0.05	0.09	-0.57	0.5760	0.96	
		vs Geo.	20 (17.5)	15 (35)										
HL_darkmagenta	130	vs Amb.	9 (6)	11 (26)	0.09	0.13	0.69	0.5010	-0.05	0.09	-0.49	0.6330	0.97	
		vs Geo.	10 (6)	9.9 (26)										
HL_darkolivegreen	131	vs Amb.	20 (10)	5.3 (23)	-0.40	0.08	-4.89	**0.0002**	0.20	0.06	3.46	0.0039	0.37	GO:0009987 cellular process
		vs Geo.	4 (10)	18 (23)										
HL_darkorange2	1438	vs Amb.	8 (20)	33 (48)	-0.02	0.13	-0.16	0.8760	0.01	0.09	0.11	0.9120	1.00	
		vs Geo.	7 (20)	32 (48)										
HL_darkslateblue	821	vs Amb.	14 (5)	20 (63)	0.18	0.12	1.44	0.1720	-0.09	0.09	-1.02	0.3260	0.87	
		vs Geo.	17 (5)	21 (63)										
HL_floralwhite	281	vs Amb.	22 (13)	6.4 (35)	0.19	0.12	1.55	0.1440	-0.10	0.09	-1.09	0.2930	0.85	
		vs Geo.	22 (13)	9 (35)										
HL_greenyellow	265	vs Amb.	13 (7)	18 (37)	0.25	0.12	2.20	**0.0454**	-0.13	0.08	-1.55	0.1427	0.74	GO:0008152 metabolic process
		vs Geo.	2 (7)	32 (37)										
HL_grey60	324	vs Amb.	7 (14)	20 (34)	-0.18	0.13	-1.41	0.1790	0.09	0.09	1.00	0.3350	0.88	
		vs Geo.	10 (14)	19 (34)										
HL_lightcyan	2794	vs Amb.	11 (22)	34 (39)	-0.25	0.12	-2.18	**0.0465**	0.13	0.08	1.54	0.1450	0.75	GO:0051179 localization
		vs Geo.	10 (22)	34 (39)										
HL_maroon	48	vs Amb.	11 (15)	6.8 (12)	0.22	0.12	1.79	0.0946	-0.11	0.09	-1.27	0.2255	0.81	
		vs Geo.	18 (15)	4.8 (12)										
HL_navajowhite2	57	vs Amb.	8 (9)	6.8 (16)	0.14	0.13	1.06	0.3070	-0.07	0.09	-0.75	0.4650	0.93	
		vs Geo.	18 (9)	5.2 (16)										
HL_orange	335	vs Amb.	21 (17.5)	5.1 (29)	0.09	0.13	0.69	0.5020	-0.05	0.09	-0.49	0.6340	0.97	
		vs Geo.	22 (17.5)	7.5 (29)										
HL_paleturquoise	137	vs Amb.	4 (2)	18 (28)	0.10	0.13	0.77	0.4540	-0.05	0.09	-0.55	0.5950	0.96	
		vs Geo.	1 (2)	25 (28)										
HL_plum1	120	vs Amb.	16 (8)	9.1 (25)	-0.15	0.13	-1.20	0.2510	0.08	0.09	0.85	0.4120	0.91	
		vs Geo.	13 (8)	11 (25)										
HL_purple	268	vs Amb.	20 (3)	5.6 (39)	-0.13	0.13	-0.97	0.3460	0.06	0.09	0.69	0.5020	0.94	
		vs Geo.	16 (3)	11 (39)										
HL_red	427	vs Amb.	23 (21)	8.8 (31)	-0.21	0.12	-1.69	0.1130	0.10	0.09	1.20	0.2520	0.83	
		vs Geo.	15 (21)	18 (31)										
HL_salmon	261	vs Amb.	17 (11.5)	10 (34)	-0.20	0.12	-1.65	0.1210	0.10	0.09	1.17	0.2630	0.84	
		vs Geo.	12 (11.5)	16 (34)										
HL_salmon4	60	vs Amb.	1 (1)	11 (17)	-0.04	0.13	-0.34	0.7430	0.02	0.09	0.24	0.8160	0.99	
		vs Geo.	3 (1)	13 (17)										
HL_violet	607	vs Amb.	18 (19)	15 (46)	0.29	0.11	2.68	**0.0181**	-0.15	0.08	-1.89	0.0793	0.66	GO:0008152 metabolic process
		vs Geo.	17 (19)	23 (46)										
HL_grey	*21*	*vs Amb.*	*23 (24)*	*-0.077 (-2.4)*										
		vs Geo.	*24 (24)*	*-0.13 (-2.4)*										
HL_gold		*vs Amb.*	*15 (23)*	*31 (2.9)*										
		vs Geo.	*20 (23)*	*30 (2.9)*										

Liver gene expression was more diverse in geothermal fish than ambient, with a gene expression clustering into 32 modules in geothermal fish and only 18 in ambient fish. One ambient liver module was not preserved in geothermal fish, and five geothermal liver modules were not preserved in ambient fish. Divergent modules between geothermal and ambient livers were found to be related to a range of biological processes, including response to stimulus (GO:0050896) (specifically steroid hormone and lipid), RNA splicing (GO:0008380), negative regulation of neuron apoptotic process (GO:0043524) and regulation of microtubule cytoskeleton organization (GO:0070507).

3.3.3 Divergence in gene expression plasticity between geothermal and ambient fish

3.3.3.1 Differential gene expression plasticity in geothermal and ambient sticklebacks

I tested for divergent gene expression plasticity in geothermal and ambient sticklebacks by comparing genes that were DE between rearing temperatures. The brains of geothermal fish were found to exhibit a much

greater number of plastic genes than found in ambient fish (Table 3-2,

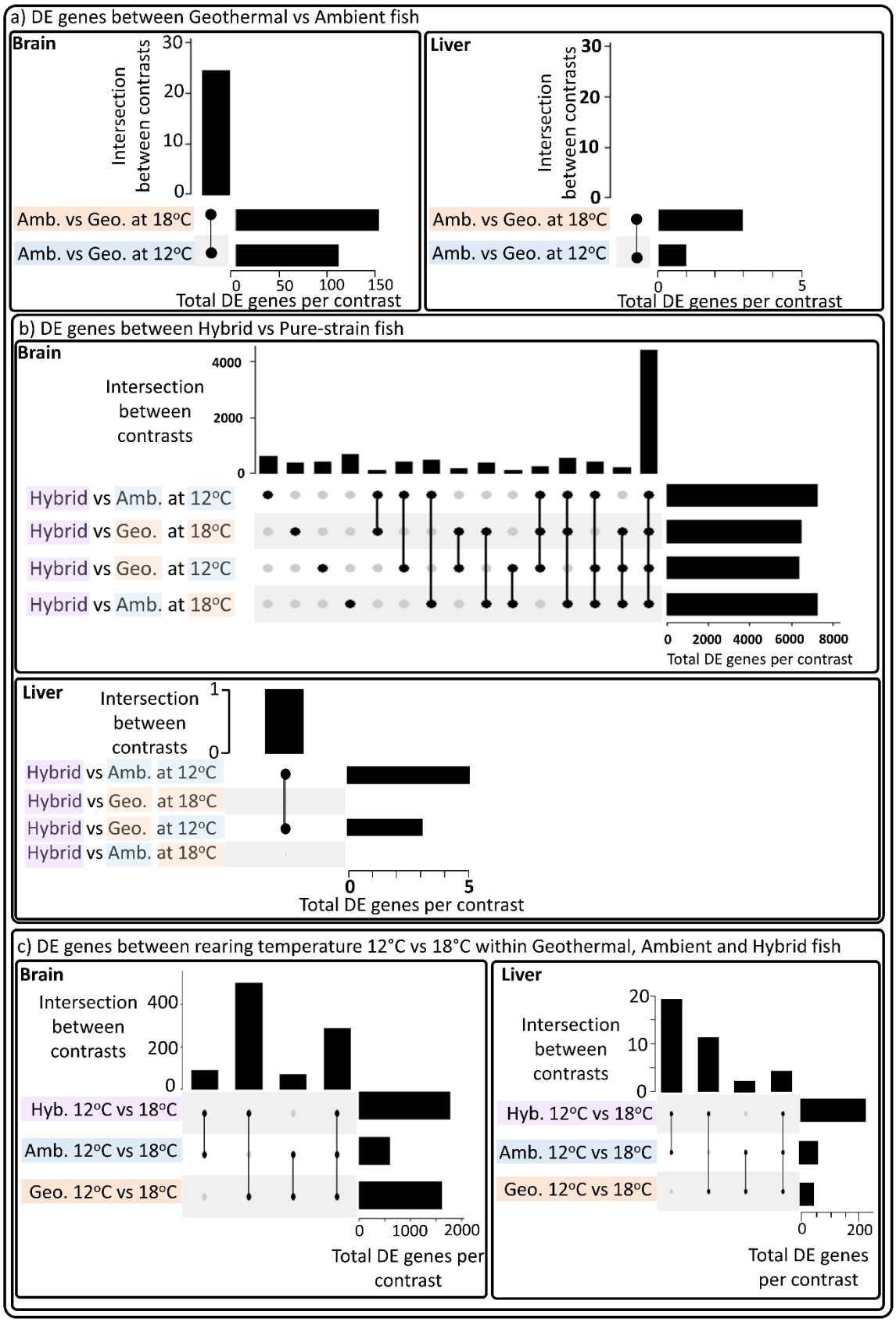


Figure 3-3c). The unique plastic responses of both geothermal and ambient fish were most strongly enriched for metabolic processes (GO:0008152). However, the metabolic processes involved in the divergent plastic responses of

geothermal and ambient fish differed. The ambient fish unique plastic response primarily involved sterol biosynthetic processes (GO:0016126), while the geothermal fish unique plastic response primarily involved organophosphate metabolic process (GO:0019637) (Table 3-6).

Table 3-5 Gene ontology analysis result of genes found to be plastic in the brains and livers of ambient, geothermal and hybrid fish. Pure-strain plasticity was divided into genes found to be plastic only in either ambient or geothermal fish, not both (unique plastic responses) and genes plastic in both ambient and geothermal fish (common plastic response)

Tissue	Analysis	Parent GO	GO term	Log(q)	Log(P)	Z	# genes
Brain	Unique Amb. fish plastic genes	GO:0002376 immune system process	macrophage migration (GO:1905517)	-1.30	-3.40	7.20	3
			myeloid leukocyte migration (GO:0097529)	-0.44	-2.10	4.10	3
		GO:0008152 metabolic process	sterol biosynthetic process (GO:0016126)	-5	-8.1	12	7
			cholesterol metabolic process (GO:0008203)	-4.8	-7.7	11	8
			secondary alcohol metabolic process (GO:1902652)	-4.3	-7.2	10	8
			sterol metabolic process (GO:0016125)	-4.3	-7.2	10	8
			secondary alcohol biosynthetic process (GO:1902653)	-4.2	-7	12	6
			cholesterol biosynthetic process (GO:0006695)	-4.2	-7	12	6
			steroid metabolic process (GO:0008202)	-4	-6.8	8.8	9
			steroid biosynthetic process (GO:0006694)	-3.3	-6	8.8	7
			farnesyl diphosphate metabolic process (GO:0045338)	-2.8	-5.4	14	3
			isoprenoid biosynthetic process (GO:0008299)	-2.4	-5.1	8.7	5
			lipid biosynthetic process (GO:0008610)	-2.2	-4.7	5.4	14
			organic hydroxy compound metabolic process (GO:1901615)	-2.2	-4.7	6	10
			organic hydroxy compound biosynthetic process (GO:1901617)	-2.1	-4.6	6.6	7
			alcohol biosynthetic process (GO:0046165)	-2	-4.4	6.7	6
			isoprenoid metabolic process (GO:0006720)	-1.9	-4.3	6.5	6
			alcohol metabolic process (GO:0006066)	-1.8	-4.1	5.7	8
			terpenoid biosynthetic process (GO:0016114)	-1.8	-4.1	9.5	3
			lipid metabolic process (GO:0006629)	-1.2	-3.3	3.9	18
			terpenoid metabolic process (GO:0006721)	-1.2	-3.3	6	4
			small molecule biosynthetic process (GO:0044283)	-1.1	-3.1	4.2	10
		GO:0032501 multicellular organismal process	epithelial cell differentiation involved in kidney development (GO:0035850)	-0.46	-2.20	4.20	3
			cell differentiation involved in kidney development (GO:0061005)	-0.46	-2.20	4.20	3
		GO:0032502 developmental process	regulation of cell differentiation (GO:0045595)	-0.41	-2.10	3.00	10
		GO:0051179 localization	lipid transport (GO:0006869)	-0.40	-2.10	3.20	6
Brain	Unique Geo. fish plastic genes	GO:0008152 metabolic process	organophosphate metabolic process (GO:0019637)	-1.7	-4	4	59
			carbohydrate metabolic process (GO:0005975)	-1.6	-3.9	4.2	35
			nucleobase-containing small molecule metabolic process (GO:0055086)	-1.5	-3.7	4	38
			organic acid metabolic process (GO:0006082)	-1.3	-3.5	3.7	55
			oxoacid metabolic process (GO:0043436)	-1.3	-3.4	3.7	54
			generation of precursor metabolites and energy (GO:0006091)	-1.3	-3.5	3.9	28
			carboxylic acid metabolic process (GO:0019752)	-1.2	-3.2	3.5	53
			nucleoside phosphate metabolic process (GO:0006753)	-1.2	-3.4	3.8	32
			tRNA metabolic process (GO:0006399)	-0.99	-3	3.5	23
			aerobic respiration (GO:0009060)	-0.92	-2.9	3.6	15
			mRNA processing (GO:0006397)	-0.81	-2.7	3.2	35
			carbohydrate catabolic process (GO:0016052)	-0.81	-2.7	3.5	12
			nucleotide metabolic process (GO:0009117)	-0.78	-2.7	3.2	29
			prostaglandin metabolic process (GO:0006693)	-0.78	-2.7	4.3	4
			prostanoid metabolic process (GO:0006692)	-0.78	-2.7	4.3	4
			icosanoid biosynthetic process (GO:0046456)	-0.78	-2.7	4.3	4
			methylglyoxal metabolic process (GO:0009438)	-0.68	-2.5	4.5	3
			isocitrate metabolic process (GO:0006102)	-0.68	-2.5	4.5	3
			tRNA aminoacylation (GO:0043039)	-0.67	-2.5	3.4	10
			long-chain fatty acid metabolic process (GO:0001676)	-0.66	-2.5	3.8	5
			energy derivation by oxidation of organic compounds (GO:0015980)	-0.61	-2.4	3.1	19
			amino acid activation (GO:0043038)	-0.61	-2.4	3.3	10
			amino sugar biosynthetic process (GO:0046349)	-0.6	-2.4	3.9	4
			monosaccharide metabolic process (GO:0005996)	-0.58	-2.4	3.2	12
			hexose metabolic process (GO:0019318)	-0.58	-2.4	3.2	11
			icosanoid metabolic process (GO:0006690)	-0.54	-2.3	3.6	5
			ATP metabolic process (GO:0046034)	-0.52	-2.3	3.3	7
			mRNA metabolic process (GO:0016071)	-0.5	-2.3	2.8	39
			RNA splicing (GO:0008380)	-0.5	-2.3	2.8	29
			proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161)	-0.5	-2.3	2.9	25
			tRNA aminoacylation for protein translation (GO:0006418)	-0.48	-2.2	3.1	9
			nucleotide-sugar metabolic process (GO:0009225)	-0.48	-2.2	3.3	6
			organophosphate biosynthetic process (GO:0090407)	-0.46	-2.2	2.7	33
			nucleoside phosphate biosynthetic process (GO:1901293)	-0.46	-2.1	2.8	19
			purine ribonucleoside triphosphate metabolic process (GO:0009205)	-0.46	-2.2	3.1	8
			tricarboxylic acid metabolic process (GO:0072350)	-0.46	-2.2	3.6	4
			DNA unwinding involved in DNA replication (GO:0006268)	-0.46	-2.1	3.9	3
			prostaglandin biosynthetic process (GO:0001516)	-0.46	-2.1	3.9	3
			prostanoid biosynthetic process (GO:0046457)	-0.46	-2.1	3.9	3
			fructose 2,6-bisphosphate metabolic process (GO:0006003)	-0.46	-2.1	3.9	3
			cellular respiration (GO:0045333)	-0.43	-2.1	2.8	15
			nucleoside triphosphate metabolic process (GO:0009141)	-0.43	-2.1	3	10
			purine nucleoside triphosphate metabolic process (GO:0009144)	-0.42	-2.1	3	8
			ribonucleoside triphosphate metabolic process (GO:0009199)	-0.4	-2.1	2.9	9
			proton motive force-driven ATP synthesis (GO:0015986)	-0.37	-2	3.2	5
		GO:0009987 cellular process	ribonucleoprotein complex biogenesis (GO:0022613)	-1.40	-3.60	3.90	42
			microtubule-based movement (GO:0007018)	-0.53	-2.30	2.90	21
			cilium movement (GO:0003341)	-0.67	-2.50	3.40	10
			cilium or flagellum-dependent cell motility (GO:0001539)	-0.37	-2.00	3.20	5
			cilium-dependent cell motility (GO:0060285)	-0.37	-2.00	3.20	5
		GO:0048519 -ve reg. of biological process	-ve regulation of vasculature development (GO:1901343)	-0.54	-2.30	3.60	5
		GO:0050789 regulation of biological process	proton transmembrane transport (GO:1902600)	-1.8	-4.2	4.8	14
			regulation of mitotic spindle assembly (GO:1901673)	-0.68	-2.5	4.5	3
			regulation of spindle assembly (GO:0090169)	-0.68	-2.5	4.5	3
			regulation of microtubule cytoskeleton organization (GO:0070507)	-0.64	-2.5	3.3	11
			regulation of spindle organization (GO:0090224)	-0.46	-2.2	3.6	4
			regulation of mitotic spindle organization (GO:0060236)	-0.46	-2.2	3.6	4
		GO:0051179 localization	regulation of microtubule-based process (GO:0032886)	-0.39	-2	2.8	12
Brain	Genes plastic in both geothermal and cold (common)	GO:0008152 metabolic process	mRNA processing (GO:0006397)	-2	-4.4	5	18
			RNA processing (GO:0006396)	-1.9	-4.3	4.5	28
			RNA splicing (GO:0008380)	-1.5	-3.7	4.5	15

Tissue	Analysis	Parent GO	GO term	Log(q)	Log(P)	Z	# genes
			RNA splicing, via transesterification reactions (GO:0000375)	-1.3	-3.4	4.3	12
			mRNA splicing, via spliceosome (GO:0000398)	-1.3	-3.4	4.3	12
			RNA splicing, via transesterification reactions (GO:0000377)	-1.3	-3.4	4.3	12
			mRNA metabolic process (GO:0016071)	-1.2	-3.3	4	18
			translational initiation (GO:0006413)	-0.71	-2.6	3.9	6
			alpha-amino acid metabolic process (GO:1901605)	-0.42	-2.1	3.1	8
			carboxylic acid metabolic process (GO:0019752)	-0.39	-2	2.7	18
			oxoacid metabolic process (GO:0043436)	-0.37	-2	2.7	18
		GO:0009987 cellular process	microtubule-based process (GO:0007017)	-1.10	-3.10	3.80	18
			microtubule-based movement (GO:0007018)	-0.81	-2.70	3.70	10
			cilium movement (GO:0003341)	-0.58	-2.40	3.80	5
		GO:0022414 reproductive process	germ cell development (GO:0007281)	-0.40	-2.10	3.30	5
		GO:0042592 homeostatic process	intracellular iron ion homeostasis (GO:0006879)	-0.49	-2.20	4.30	3
		GO:0048518 +ve regulation of biological process	+ve regulation of transmembrane transport (GO:0034764)	-0.55	-2.30	4.50	3
		GO:0050896 response to stimulus	retinoic acid receptor signalling pathway (GO:0048384)	-0.49	-2.20	4.30	3
		GO:0065007 biological regulation	protein stabilization (GO:0050821)	-0.49	-2.20	4.30	3
Brain	**Genes plastic in Hybrid fish**	**GO:0008152 metabolic process**	amide biosynthetic process (GO:0043604)	-8.7	-12	8.1	88
			peptide biosynthetic process (GO:0043043)	-8.1	-12	7.9	75
			translation (GO:0006412)	-8.1	-12	7.9	74
			amide metabolic process (GO:0043603)	-6.8	-10	7.1	100
			peptide metabolic process (GO:0006518)	-6.8	-10	7.2	78
			sterol biosynthetic process (GO:0016126)	-5.7	-8.9	8.1	14
			secondary alcohol biosynthetic process (GO:1902653)	-4.8	-7.8	7.6	12
			cholesterol biosynthetic process (GO:0006695)	-4.8	-7.8	7.6	12
			secondary alcohol metabolic process (GO:1902652)	-4.4	-7.3	6.8	18
			cholesterol metabolic process (GO:0008203)	-4	-6.8	6.6	16
			steroid biosynthetic process (GO:0006694)	-3.2	-5.9	6	16
			sterol metabolic process (GO:0016125)	-3	-5.7	5.8	16
			cytoplasmic translation (GO:0002181)	-2.5	-5.2	5.4	17
			RNA splicing (GO:0008380)	-2.4	-5	4.8	45
			RNA splicing, via transesterification reactions with bulged adenosine as nucleophile (GO:0000377)	-2.4	-4.9	4.8	36
			RNA splicing, via transesterification reactions (GO:0000375)	-2.4	-4.9	4.8	36
			mRNA splicing, via spliceosome (GO:0000398)	-2.4	-4.9	4.8	36
			steroid metabolic process (GO:0008202)	-2.2	-4.7	5	19
			mRNA processing (GO:0006397)	-2.1	-4.6	4.5	50
			organic hydroxy compound biosynthetic process (GO:1901617)	-2.1	-4.6	4.9	19
			alcohol biosynthetic process (GO:0046165)	-1.8	-4.2	4.7	15
			mRNA metabolic process (GO:0016071)	-1.7	-4	4	56
			translational initiation (GO:0006413)	-1.6	-3.9	4.4	17
			isoprenoid biosynthetic process (GO:0008299)	-1.5	-3.7	4.6	9
			thioester biosynthetic process (GO:0035384)	-1.3	-3.5	4.5	8
			acyl-CoA biosynthetic process (GO:0071616)	-1.3	-3.5	4.5	8
			acetyl-CoA metabolic process (GO:0006084)	-1.3	-3.4	4.5	7
			organophosphate biosynthetic process (GO:0090407)	-1.2	-3.3	3.6	46
			nucleoside phosphate metabolic process (GO:0006753)	-1.2	-3.3	3.6	39
			alcohol metabolic process (GO:0006066)	-1.2	-3.4	3.8	23
			thioester metabolic process (GO:0035383)	-1.2	-3.4	4.1	13
			acyl-CoA metabolic process (GO:0006637)	-1.2	-3.4	4.1	13
			polyamine biosynthetic process (GO:0006596)	-1.2	-3.3	4.7	5
			lipid biosynthetic process (GO:0008610)	-1.1	-3.1	3.5	47
			ribonucleoside bisphosphate metabolic process (GO:0033875)	-1.1	-3.1	3.8	14
			nucleoside bisphosphate metabolic process (GO:0033865)	-1.1	-3.1	3.8	14
			purine nucleoside bisphosphate metabolic process (GO:0034032)	-1.1	-3.1	3.8	14
			nucleotide metabolic process (GO:0009117)	-1	-3	3.4	37
			biogenic amine biosynthetic process (GO:0042401)	-1	-3	4.2	6
			amine biosynthetic process (GO:0009309)	-1	-3	4.2	6
			ribose phosphate metabolic process (GO:0019693)	-0.92	-2.9	3.4	29
			tricarboxylic acid cycle (GO:0006099)	-0.85	-2.8	3.7	9
			organic hydroxy compound metabolic process (GO:1901615)	-0.84	-2.8	3.3	27
			ribonucleotide metabolic process (GO:0009259)	-0.81	-2.7	3.2	28
			acetyl-CoA biosynthetic process from pyruvate (GO:0006086)	-0.81	-2.7	4.6	3
			isopentenyl diphosphate biosynthetic process (GO:0009240)	-0.81	-2.7	4.6	3
			isopentenyl diphosphate metabolic process (GO:0046490)	-0.81	-2.7	4.6	3
			farnesyl diphosphate metabolic process (GO:0045338)	-0.81	-2.7	4.6	3
			purine nucleotide metabolic process (GO:0006163)	-0.78	-2.7	3.2	30
			tricarboxylic acid metabolic process (GO:0072350)	-0.74	-2.6	3.9	5
			polyamine metabolic process (GO:0006595)	-0.74	-2.6	3.9	5
			nucleoside bisphosphate biosynthetic process (GO:0033866)	-0.73	-2.6	3.6	8
			purine nucleoside bisphosphate biosynthetic process (GO:0034033)	-0.73	-2.6	3.6	8
			ribonucleoside bisphosphate biosynthetic process (GO:0034030)	-0.73	-2.6	3.6	8
			purine-containing compound metabolic process (GO:0072521)	-0.58	-2.4	2.9	32
			small molecule biosynthetic process (GO:0044283)	-0.54	-2.3	2.8	36
			biogenic amine metabolic process (GO:0006576)	-0.53	-2.3	3.2	8
			ribonucleotide biosynthetic process (GO:0009260)	-0.48	-2.2	2.9	20
			acetyl-CoA biosynthetic process (GO:0006085)	-0.48	-2.2	3.6	4
			long-chain fatty-acyl-CoA biosynthetic process (GO:0035338)	-0.48	-2.2	3.6	4
			amine metabolic process (GO:0009308)	-0.46	-2.2	3.1	8
			ribose phosphate biosynthetic process (GO:0046390)	-0.44	-2.1	2.8	20
			nucleotide biosynthetic process (GO:0009165)	-0.41	-2.1	2.7	23
			monocarboxylic acid metabolic process (GO:0032787)	-0.39	-2	2.6	34
			purine ribonucleotide metabolic process (GO:0009150)	-0.38	-2	2.6	23
			nucleoside phosphate biosynthetic process (GO:1901293)	-0.38	-2	2.6	23
			proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161)	-0.37	-2	2.6	30
		GO:0009987 cellular process	cilium movement (GO:0003341)	-3	-5.7	5.8	17
			protein-RNA complex organization (GO:0071826)	-2.4	-5	4.9	31
			microtubule-based movement (GO:0007018)	-2.1	-4.5	4.6	32
			protein-RNA complex assembly (GO:0022618)	-2	-4.4	4.5	29
			ribonucleoprotein complex biogenesis (GO:0022613)	-1.9	-4.2	4.2	54
			cilium-dependent cell motility (GO:0060285)	-1.5	-3.8	4.8	8
			cilium or flagellum-dependent cell motility (GO:0001539)	-1.5	-3.8	4.8	8
			microtubule-based process (GO:0007017)	-1.3	-3.5	3.7	56
			cilium movement involved in cell motility (GO:0060294)	-1.3	-3.4	4.5	7
			microtubule bundle formation (GO:0001578)	-1.1	-3.2	4	10
			axoneme assembly (GO:0035082)	-1.1	-3.1	4	9
			cilium organization (GO:0044782)	-0.87	-2.8	3.3	36
			motile cilium assembly (GO:0044458)	-0.82	-2.8	3.9	6
			ribosomal small subunit biogenesis (GO:0042274)	-0.74	-2.6	3.3	14
			cilium assembly (GO:0060271)	-0.68	-2.5	3	33

Tissue	Analysis	Parent GO	GO term	Log(q)	Log(P)	Z	# genes
			plasma membrane bounded cell projection assembly (GO:0120031)	-0.67	-2.5	3	35
			microtubule cytoskeleton organization (GO:0000226)	-0.53	-2.3	2.8	35
			cell projection assembly (GO:0030031)	-0.43	-2.1	2.6	35
		GO:0022414 reproductive process	flagellated sperm motility (GO:0030317)	-1.20	-3.30	4.70	5
			sperm motility (GO:0097722)	-1.20	-3.30	4.70	5
			sperm axoneme assembly (GO:0007288)	-0.81	-2.70	4.60	3
			sperm flagellum assembly (GO:0120316)	-0.46	-2.20	3.80	3
		GO:0032501 multicellular organismal process	late endosome to vacuole transport (GO:0045324)	-1.3	-3.5	4.4	9
		GO:0051179 localization	endosome transport via multivesicular body sorting pathway (GO:0032509)	-1.3	-3.5	4.4	9
			multivesicular body sorting pathway (GO:0071985)	-1.3	-3.5	4.4	9
			endosomal transport (GO:0016197)	-1.2	-3.2	3.7	25
			vacuolar transport (GO:0007034)	-1.1	-3.1	3.7	18
			proton transmembrane transport (GO:1902600)	-0.6	-2.4	3.2	13
			epithelial cilium movement involved in extracellular fluid movement (GO:0003351)	-0.58	-2.4	3.4	7
			extracellular transport (GO:0006858)	-0.58	-2.4	3.4	7
			microtubule-based transport (GO:0099111)	-0.55	-2.3	3	17
			spinal cord oligodendrocyte cell differentiation (GO:0021529)	-0.46	-2.2	3.8	3
			cerebrospinal fluid circulation (GO:0090660)	-0.46	-2.2	3.8	3
Liver	**Geo. plastic**	**GO:0008152 metabolic process**	regulation of catabolic process (GO:0009894)	0	-2.30	3.90	4
			dephosphorylation (GO:0016311)	0	-2.00	3.80	3
Liver	**Hybrid Plastic**	**GO:0008152 metabolic process**	serine family amino acid biosynthetic process (GO:0009070)	0	-2.70	5.50	3
			heme metabolic process (GO:0042168)	0	-2.20	4.20	3
			porphyrin-containing compound metabolic process (GO:0006778)	0	-2.10	4.00	3
			serine family amino acid metabolic process (GO:0009069)	0	-2.00	3.90	3
		GO:0032501 multicellular organismal process	circulatory system process (GO:0003013)	0	-2.60	4.00	6
			blood circulation (GO:0008015)	0	-2.60	4.00	6
		GO:0065007 biological regulation	regulation of membrane potential (GO:0042391)	0	-2.40	4.20	4

Gene expression in the liver was found to be much less plastic than in the brain. Additionally, geothermal, and ambient fish did not differ greatly in the amount of plasticity found in the liver, with plastic responses involving a similar

number of genes (Table 3-2,

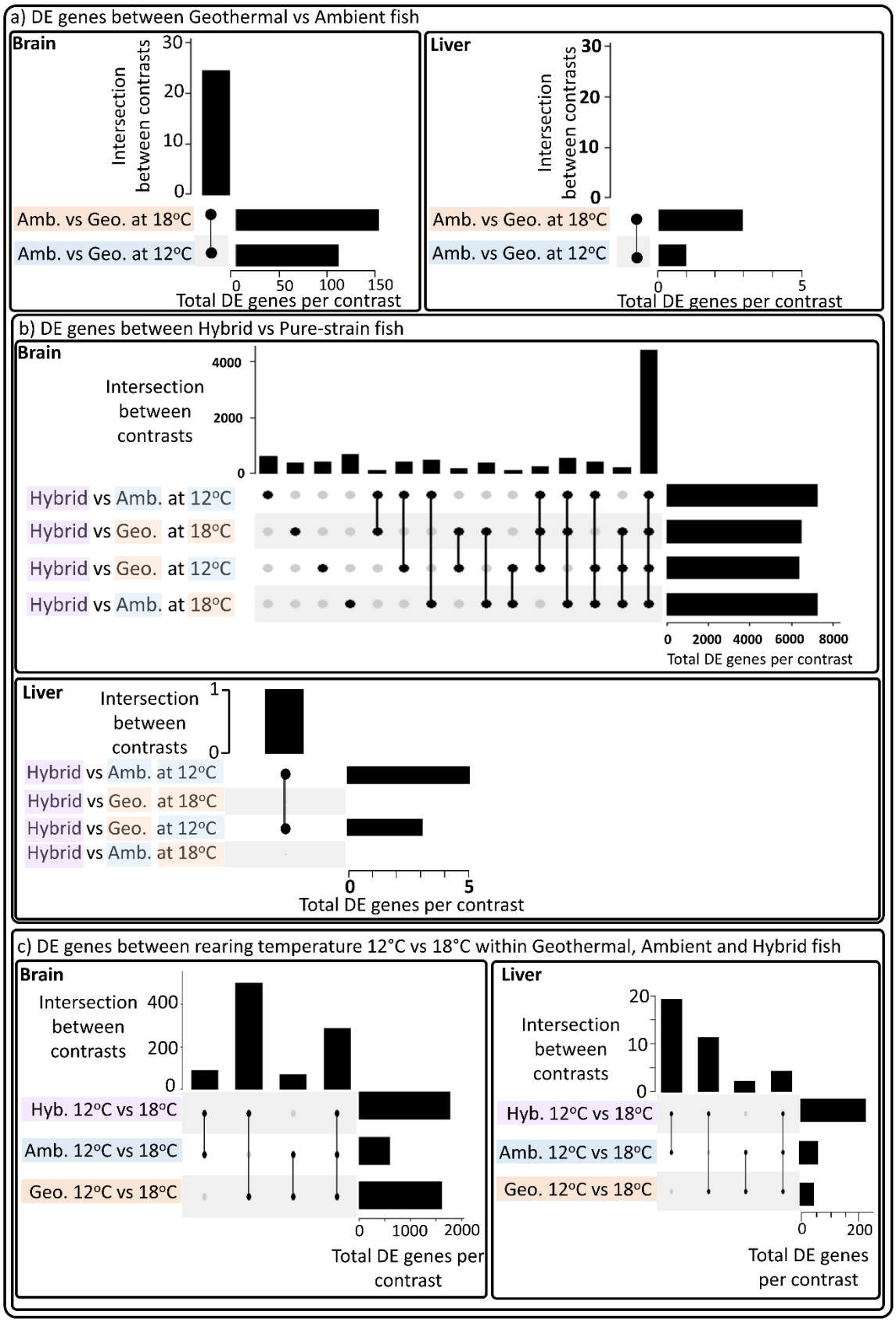


Figure 3-3c). The plastic response of the livers of geothermal fish was found to be enriched for regulation of catabolic process (GO:0009894), however, no gene ontology was found to be enriched in the plastic response of ambient fish.

3.3.3.2 Divergence of gene module plasticity between geothermal and ambient fish

I tested for divergence in the plasticity of modules of co-expressed genes between geothermal and ambient sticklebacks. First, I tested for evidence of a relationship between module co-expression and temperature. In the brain, the co-expression of nine geothermal modules and ten ambient modules was found to respond to rearing temperature (Table 3-4). In the liver, four geothermal fish modules were found to respond to rearing temperature (Table 3-4).

Next, I tested for divergence in plasticity by assessing whether plastic geothermal modules corresponded to plastic ambient modules via a consensus network. Of the nine geothermal modules found to be plastic in the brain, I found four that did not correspond with ambient plastic modules. Three of these modules (*GB_brown4*, *GB_darkorange* and *GB_thistle2*) corresponded with ambient modules not found to be plastic and one module (*GB_ivory*) did not clearly correspond with any ambient modules (Figure 3-6). Seven ambient fish modules found to be plastic in ambient fish did not correspond to plastic geothermal brain modules. One of these modules (*AB_green*) corresponded to a geothermal module not found to be plastic and the remaining six modules did not correspond to any geothermal modules (*AB_darkorange, AB_floralwhite, AB_lightcyan, AB_lightsteelblue1, AB_orange* and *AB_skyblue*) (Figure 3-6). Divergent module plasticity in the brain involves an array of gene ontologies (Table 4).

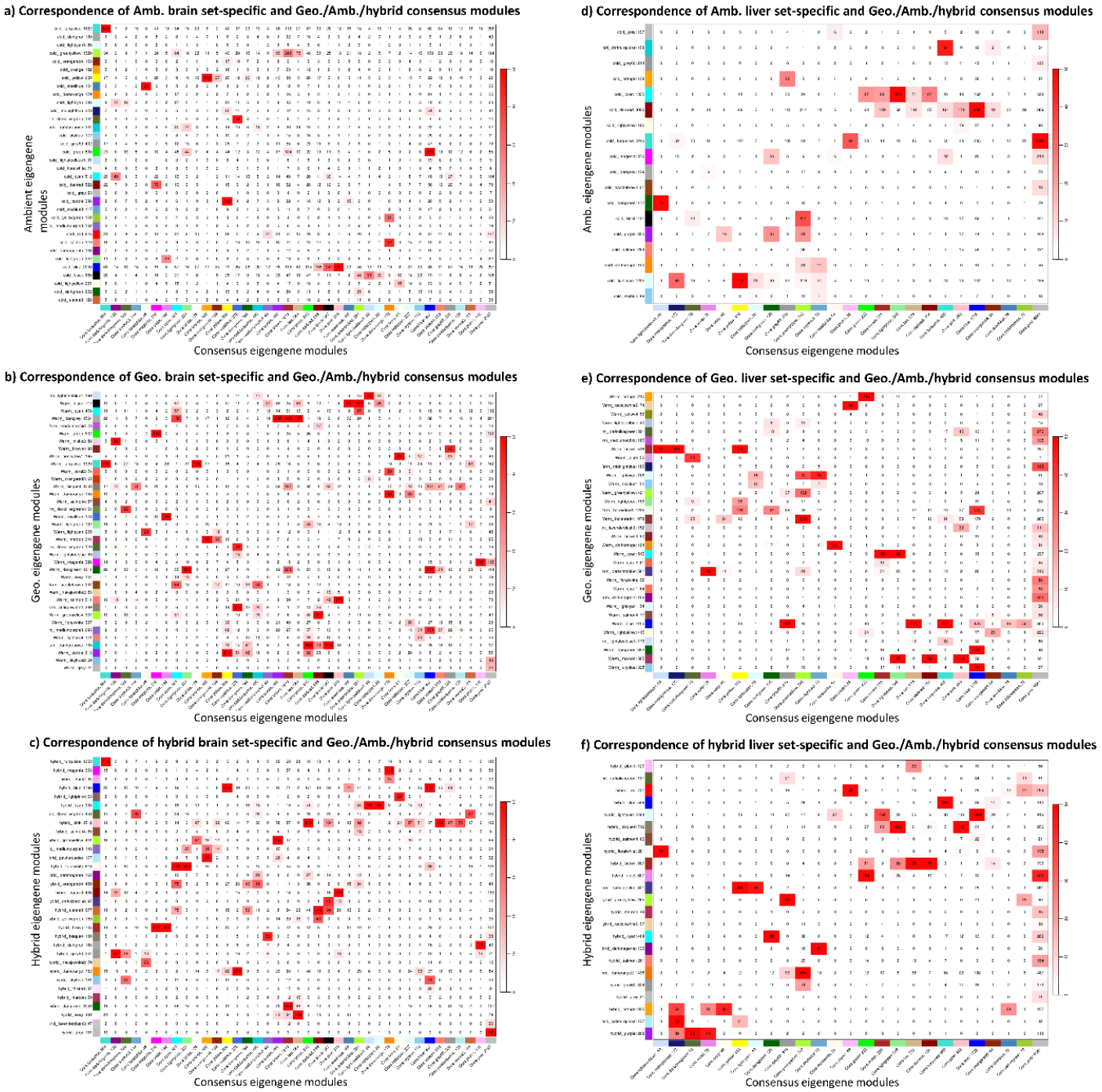


Figure 3-6 Correspondence of fish type specific eigengene modules and the geothermal/ambient/hybrid consensus modules in brain (left panels) and liver (right panels)

As I did not find any plastic modules in the livers of ambient fish, the four plastic modules in the geothermal liver dataset are divergently plastic (*GL_coral1*, *GL_darkmagenta*, *GL_lightsteelblue* and *GL_mediumorchid*) (Table 3-4).

3.3.4 Disruption of gene expression in hybrids

3.3.4.1 Differential expression between hybrid and parental populations

I tested for DE between hybrid fish and pure-strain fish representing the parental populations of the hybrids. I compared hybrid gene expression to each pure-strain type fish at each rearing temperature. Differential expression between hybrids and pure-strain fish was found to be much more prominent in the brain than in the liver. A very large number of genes (ranging from 6023-6986) were found to be DE in each comparison between hybrid and pure-strain brains at each rearing temperature (Table 3-2). This result suggests a high degree of mis-expression in hybrid fish, specifically in the brain. Hybrid differential expression in the brain was found to be consistent across both temperature and the pure-strain used for the comparison, with a large proportion (4215) of these genes found to be DE in all four comparisons (Table

3-2,

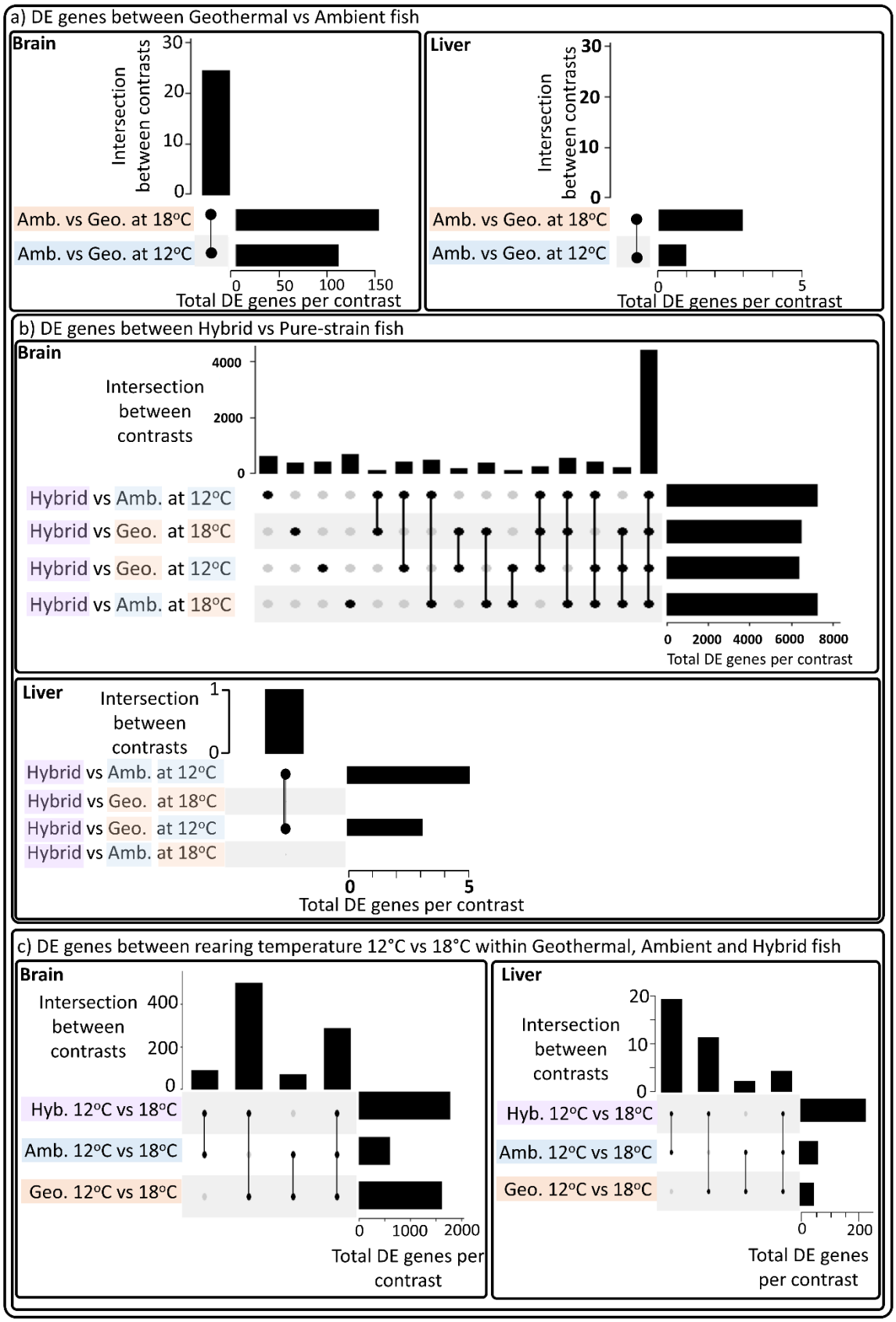


Figure 3-3b). The misexpression of genes in hybrids covers a wide array of gene ontologies, ((Previous page) Figure 3-4b, Table 3-5).

Table 3-6 Gene ontology enrichment of genes found to be differentially expressed in hybrids compared to both pure-strain types at both rearing temperatures.

Parent GO term	GO term	Log(q)	Log(P)	Z	# genes
GO:0008152 metabolic process	peptide biosynthetic process (GO:0043043)	-9.7	-13	8.7	62
	translation (GO:0006412)	-9.7	-13	8.6	61
	peptide metabolic process (GO:0006518)	-9.7	-13	8.5	67
	amide biosynthetic process (GO:0043604)	-6.5	-9.3	7	63
	amide metabolic process (GO:0043603)	-5.5	-8.3	6.4	73
	cytoplasmic translation (GO:0002181)	-3.9	-6.6	6.7	16
	respiratory electron transport chain (GO:0022904)	-2.7	-5.3	5.7	15
	aerobic electron transport chain (GO:0019646)	-2.6	-5.2	5.7	13
	mitochondrial ATP synthesis coupled electron transport (GO:0042775)	-2.5	-5	5.6	13
	oxidative phosphorylation (GO:0006119)	-2.4	-4.9	5.5	14
	electron transport chain (GO:0022900)	-2.4	-4.9	5.4	15
	ATP synthesis coupled electron transport (GO:0042773)	-2.4	-4.9	5.5	13
	mitochondrial electron transport, cytochrome c to oxygen (GO:0006123)	-1.6	-3.9	5.4	6
	cellular respiration (GO:0045333)	-1.1	-3.3	4	17
	aerobic respiration (GO:0009060)	-1	-3.2	3.9	15
	mitochondrial electron transport, ubiquinol to cytochrome c (GO:0006122)	-0.99	-3.1	5.1	4
	protein demethylation (GO:0006482)	-0.4	-2.3	3.8	4
	protein dealkylation (GO:0008214)	-0.4	-2.3	3.8	4
	adenosine to inosine editing (GO:0006382)	-0.36	-2.2	4.1	3
	demethylation (GO:0070988)	-0.29	-2.2	3.4	5
	energy derivation by oxidation of organic compounds (GO:0015980)	-0.22	-2.1	2.8	17
GO:0009987 cellular process	cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	-2.3	-4.8	5.2	17
	cell-cell adhesion (GO:0098609)	-1.3	-3.5	4	24
	mitochondrial respiratory chain complex assembly (GO:0033108)	-1	-3.3	4.2	10
	NADH dehydrogenase complex assembly (GO:0010257)	-0.99	-3.2	4.4	7
	mitochondrial respiratory chain complex I assembly (GO:0032981)	-0.99	-3.2	4.4	7
	synapse organization (GO:0050808)	-0.73	-2.8	3.5	16
	cell adhesion (GO:0007155)	-0.64	-2.7	3.2	34
	protein-RNA complex assembly (GO:0022618)	-0.63	-2.7	3.3	19
	ribosomal small subunit assembly (GO:0000028)	-0.61	-2.6	4.1	5
	synaptic membrane adhesion (GO:0099560)	-0.56	-2.5	4.2	4
	ribosomal small subunit biogenesis (GO:0042274)	-0.54	-2.5	3.4	11
	ribosome assembly (GO:0042255)	-0.53	-2.5	3.5	8
	protein-RNA complex organization (GO:0071826)	-0.53	-2.5	3.1	19
	non-membrane-bounded organelle assembly (GO:0140694)	-0.44	-2.4	3	21
	homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	-0.43	-2.4	3.2	10
	microtubule-based process (GO:0007017)	-0.43	-2.4	2.9	38
	cell recognition (GO:0008037)	-0.37	-2.3	3.3	7
	cytoplasmic microtubule organization (GO:0031122)	-0.36	-2.3	3.4	6
GO:0032501 multicellular organismal process	semaphorin-plexin signalling pathway involved in neuron projection guidance (GO:1902285)	-1.7	-4.1	5.9	5
	semaphorin-plexin signalling pathway involved in axon guidance (GO:1902287)	-1.7	-4.1	5.9	5
	neuron recognition (GO:0008038)	-0.99	-3.2	4.4	7
	neuromuscular process controlling balance (GO:0050885)	-0.99	-3.1	5.1	4
	neuron projection guidance (GO:0097485)	-0.77	-2.9	3.4	25
	axon guidance (GO:0007411)	-0.77	-2.9	3.4	25
	embryo development ending in birth or egg hatching (GO:0009792)	-0.72	-2.8	3.2	44
	chordate embryonic development (GO:0043009)	-0.6	-2.6	3.1	43
	neuronal action potential (GO:0019228)	-0.56	-2.5	4.2	4
	neuromuscular process (GO:0050905)	-0.53	-2.5	3.5	8
	transmission of nerve impulse (GO:0019226)	-0.44	-2.4	3.6	6
	neuron projection development (GO:0031175)	-0.44	-2.4	2.9	42
	regulation of axonogenesis (GO:0050770)	-0.21	-2.1	2.9	11
	neuron projection morphogenesis (GO:0048812)	-0.21	-2.1	2.6	35
	embryonic pectoral fin morphogenesis (GO:0035118)	-0.2	-2	3.2	5
GO:0032502 developmental process	regulation of cell shape (GO:0008360)	-0.91	-3	4	10
	hemopoiesis (GO:0030097)	-0.73	-2.8	3.3	40
	regulation of cell morphogenesis (GO:0022604)	-0.68	-2.7	3.6	11
	myeloid cell differentiation (GO:0030099)	-0.65	-2.7	3.3	22
	plasma membrane bounded cell projection morphogenesis (GO:0120039)	-0.21	-2.1	2.6	35
	cell projection morphogenesis (GO:0048858)	-0.2	-2	2.6	35
	myeloid cell development (GO:0061515)	-0.2	-2	2.9	10
GO:0042592 homeostatic process	release of sequestered calcium ion into cytosol by endoplasmic reticulum (GO:1903514)	-0.61	-2.6	4.7	3
	release of sequestered calcium ion into cytosol by sarcoplasmic reticulum (GO:0014808)	-0.61	-2.6	4.7	3
	erythrocyte differentiation (GO:0030218)	-0.6	-2.6	3.3	15
	myeloid cell homeostasis (GO:0002262)	-0.6	-2.6	3.3	16
	erythrocyte homeostasis (GO:0034101)	-0.56	-2.5	3.3	15
	homeostasis of number of cells (GO:0048872)	-0.36	-2.2	2.9	17
	erythrocyte development (GO:0048821)	-0.21	-2.1	3	8
GO:0048518 positive regulation of biological process	positive regulation of axonogenesis (GO:0050772)	-0.75	-2.9	4	7
GO:0048519 negative regulation of biological process	negative regulation of sodium ion transport (GO:0010766)	-0.36	-2.2	4.1	3
	synaptic signalling (GO:0099536)	-0.91	-3	3.6	27
	chemical synaptic transmission (GO:0007268)	-0.47	-2.4	3	24
	anterograde trans-synaptic signalling (GO:0098916)	-0.47	-2.4	3	24
	trans-synaptic signalling (GO:0099537)	-0.42	-2.3	2.9	24
GO:0050896 response to stimulus	response to auditory stimulus (GO:0010996)	-0.99	-3.1	5.1	4
	semaphorin-plexin signalling pathway (GO:0071526)	-0.44	-2.4	3.4	7
	response to mechanical stimulus (GO:0009612)	-0.2	-2	3.1	6
GO:0051179 localization	calcium ion transmembrane transport (GO:0070588)	-2.8	-5.4	5.6	20
	monoatomic cation transmembrane transport (GO:0098655)	-2	-4.5	4.5	39
	inorganic cation transmembrane transport (GO:0098662)	-2	-4.4	4.5	39
	calcium ion transport (GO:0006816)	-2	-4.4	4.8	20
	inorganic ion transmembrane transport (GO:0098660)	-1.9	-4.3	4.3	43
	monoatomic ion transmembrane transport (GO:0034220)	-1.5	-3.8	4	43
	calcium ion transmembrane import into cytosol (GO:0097553)	-1.3	-3.5	4.6	9
	regulation of monoatomic ion transmembrane transport (GO:0034765)	-1	-3.2	3.8	20
	regulation of transport (GO:0051049)	-0.73	-2.8	3.3	38
	regulation of monoatomic ion transport (GO:0043269)	-0.7	-2.8	3.4	20
	monoatomic cation transport (GO:0006812)	-0.68	-2.7	3.2	40
	regulation of transmembrane transport (GO:0034762)	-0.67	-2.7	3.4	20
	regulation of localization (GO:0032879)	-0.66	-2.7	3.2	43
	sarcoplasmic reticulum calcium ion transport (GO:0070296)	-0.61	-2.6	4.7	3
	synaptic vesicle cycle (GO:0099504)	-0.6	-2.6	3.4	12
	vesicle-mediated transport in synapse (GO:0099003)	-0.6	-2.6	3.4	12
	regulated exocytosis (GO:0045055)	-0.56	-2.5	3.5	9
	calcium ion import across plasma membrane (GO:0098703)	-0.51	-2.5	3.8	5
	metal ion transport (GO:0030001)	-0.49	-2.4	3	34

Parent GO term	GO term	Log(q)	Log(P)	Z	# genes
	calcium-ion regulated exocytosis (GO:0017156)	-0.44	-2.4	3.6	6
	synaptic vesicle exocytosis (GO:0016079)	-0.22	-2.1	3.1	7
	regulation of protein secretion (GO:0050708)	-0.2	-2	3.2	5
GO:0065007 biological regulation	regulation of membrane potential (GO:0042391)	-0.76	-2.9	3.5	18
	action potential (GO:0001508)	-0.67	-2.7	3.9	7
	regulation of GTPase activity (GO:0043087)	-0.27	-2.1	2.9	15

3.3.4.2 Disruption of gene modules in hybrids

I assessed the disruption to the interactions between genes by testing whether hybrid gene modules were preserved in pure-strain fish. Hybrid gene expression was co-expressed in 23 modules in the liver and 32 modules in the brain. There was little disruption to co-expression in hybrid fish, as hybrid gene modules were found to be preserved in ambient and geothermal gene expression (Table 3-4).

3.3.5 Disruption of gene expression plasticity in hybrids

3.3.5.1 Differential gene expression plasticity in hybrids

The brain appeared to be more plastic in gene expression relative to the liver in hybrids across rearing temperatures. Hybrid plastic responses consisted of 1526 genes in the brain and 219 in the liver DE with rearing temperature (Table 3-2). There was overlap with pure strain fish, although this was less overall for ambient source fish as 354 genes in the brain and 23 in the liver overlapped, while in geothermal-source fish 749 in the brain and 15 in the liver

overlapped (

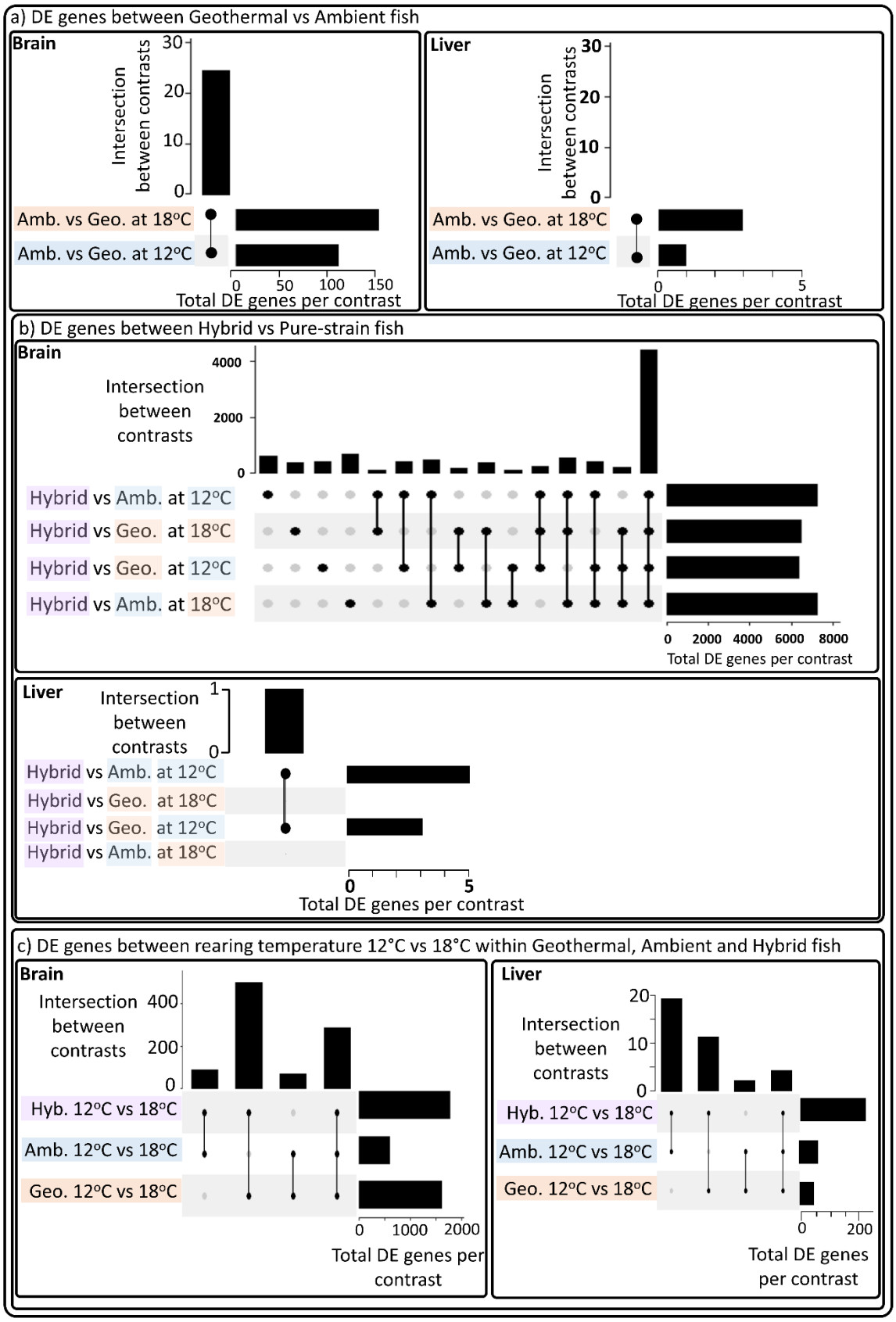


Figure 3-3c). Genes that were plastic in the brains of hybrids, but not in geothermal or ambient fish, were most strongly enriched for a variety of metabolic processes (GO:0008152).

3.3.5.2 Disruption of gene module plasticity in hybrids

Six brain and four liver hybrid modules were plastic. I found no evidence of disruption in module plasticity, as all of these modules corresponded with modules found to be plastic in geothermal, ambient or both pure-strain fish (Figure 3-6).

3.3.6 Regulatory mechanisms underlying divergence.

3.3.6.1 Inheritance modes of parental gene expression

I categorized hybrid gene expression by inheritance modes based upon how hybrid gene expression related to pure-strain gene expression. I found little evidence of significant amounts of additive inheritance in either the brain or the liver. In the brain, the additive inheritance category had the fewest genes of all the inheritance modes assessed (Table 3-7). The additive inheritance category was also among the smallest in the liver (Table 3-7).

Table 3-7 Distribution of genes across inheritance categories in at each rearing temperature in brain and liver gene expression. Hybrid gene expression was compared to geothermal and ambient gene expression and divided into five inheritance categories.

	Brain			**Liver**		
Inheritance type	*At 12oC*	*At 18oC*	*Overlapping*	*At 12oC*	*At 18oC*	*Overlapping*
Transgressive (over-expressed)	1729	1772	*1249*	5	3	2
Transgressive (under-expressed)	2180	2157	*1776*	8	3	3
Additive	20	25	*3*	1	4	0
Ambient dominance	468	477	*104*	1	4	0
Geothermal dominance	1005	1060	*293*	2	4	0

I found evidence of widespread transgressive expression in the brains of hybrid fish. Of the 13452 genes analyzed in the brain 3909 were transgressive at 12°C and 3929 were transgressive at 18°C (Table 3-7). More transgressive genes were under-expressed than over-expressed. Metabolic processes (GO:0008152) were most affected by both transgressive under- and over-expression in the brain. Pure-strain type dominant genes were relatively common in the brain, and more genes were classed as geothermal dominant than ambient dominant (Table 3-7). Geothermal dominant genes in the brain were most strongly associated with metabolic processes (GO:0008152). Ambient dominant genes in the brain were most strongly related to multicellular organismal processes (GO:0032501), particularly in terms relating to morphogenesis.

3.3.6.2 Cis and trans regulatory mechanisms

From my analysis of allele-specific expression, a small number of SNPs were found to display allele specific expression. However, this ranged from zero to four across treatment groups suggesting little evidence for cis-regulatory divergence between geothermal and ambient sticklebacks (Table 3-8). The detection of ASE depended on rearing temperature (Table 3-8) with 140 SNPs detected at 12C in the brain and 88 SNPs in the liver, while at 18°C 117 SNPs were detected for the brain and 97 SNPs for the liver had ASE. No enriched GO terms were found for either list of SNPs.

Table 3-8 ASEP workflow result, with numbers of pure-strain fish type unique SNPs, hybrid consistent heterozygous SNPs, hybrid heterozygous SNPs found to have significant ASE and the number of which were also straight type specific SNPs.

Identification of SNPs in Ambient and Geothermal fish							
Tissue		**Brain**			**Liver**		
Pure-strain fish type		Ambient	Geothermal		Ambient	Geothermal	
SNPs unique to Pure-strain type		14052 SNPs, 6885 genes	22537 SNPs, 9335 genes		7488 SNPs, 3913 genes	9998 SNPs, 5014 genes	
Assessment of SNPs for ASE at different rearing temperatures							
Tissue		**Brain**			**Liver**		
Rearing temperature		12ºC	18 ºC	overlap	12 ºC	18 ºC	overlap
Heterozygous SNPs consistent across hybrids		2484	2327	1483	362	437	246
Hybrid Het. SNPs with sig. ASE		140	117		88	97	
SNPs heterozygous in hybrids that are also straight type unique SNPs	Ambient type SNP	7			2		
	Geothermal type SNP	46			7		
SNPs with Sig. ASE in hybrids that are also Straight type unique	Ambient type SNP	1 SNP, 1 gene	0	0	1 SNP, 1 gene	0	0
	Geothermal type SNP	4 SNP, 4 gene	0	0	2 SNPs, 2 genes	2 SNPs, 2 genes	2 SNPs, 2 genes

Table 3-9 Most significantly enriched top-level GO terms, and up to five most significantly enriched child GO terms for each module. AB = ambient brain, AL = ambient liver, GB = geothermal brain, GL = geothermal liver, HB = hybrid brain and HL = hybrid liver.

module	Parent GO term	GO_term	#Genes	Log(q)	Log(P)	Z-score
AB_darkorange	GO:0051179 localization	intraciliary transport (GO:0042073)	5	-2.40	-5.10	8.80
		protein-containing complex localization (GO:0031503)	6	-1.10	-3.40	5.20
		cytoskeleton-dependent intracellular transport (GO:0030705)	6	-0.98	-3.20	4.90
		transport along microtubule (GO:0010970)	5	-0.92	-3.10	5.00
		microtubule-based transport (GO:0099111)	5	-0.58	-2.50	4.10
AB_darkred	GO:0002376 immune system process	immune system process (GO:0002376)	27	-2.20	-4.80	5.00
		immune response (GO:0006955)	13	-1.10	-3.30	4.20
		-ve reg. of leukocyte differentiation (GO:1902106)	3	-0.87	-3.00	5.90
		-ve reg. of hemopoiesis (GO:1903707)	3	-0.87	-3.00	5.90
		reg. of immune system process (GO:0002682)	14	-0.73	-2.80	3.60
AB_green	GO:0008152 metabolic process	sterol biosynthetic process (GO:0016126)	7	-1.60	-4.10	5.60
		isoprenoid biosynthetic process (GO:0008299)	7	-1.60	-4.10	5.60
		cholesterol metabolic process (GO:0008203)	9	-1.40	-3.80	5.00
		mRNA processing (GO:0006397)	28	-1.40	-3.80	4.20
		steroid biosynthetic process (GO:0006694)	9	-1.30	-3.70	4.80
AB_greenyellow	GO:0008152 metabolic process	RNA processing (GO:0006396)	80	-3.40	-6.10	5.20
		ncRNA processing (GO:0034470)	42	-1.70	-4.30	4.30
		rRNA metabolic process (GO:0016072)	28	-1.60	-4.20	4.40
		ncRNA metabolic process (GO:0034660)	50	-1.60	-4.10	4.20
		rRNA processing (GO:0006364)	26	-1.50	-4.00	4.30
AB_lightcyan	GO:0009987 cellular process	mitochondrial genome maintenance (GO:0000002)	3	-0.85	-3.10	6.30
AB_lightsteelblue1	GO:0008152 metabolic process	aerobic respiration (GO:0009060)	5	-1.50	-4.00	6.70
		cellular respiration (GO:0045333)	5	-1.20	-3.60	6.00
		energy derivation by oxidation of organic compounds (GO:0015980)	5	-0.92	-3.20	5.20
		generation of precursor metabolites and energy (GO:0006091)	5	-0.74	-2.90	4.70
		aerobic electron transport chain (GO:0019646)	3	-0.65	-2.70	5.60
AB_midnightblue	GO:0008152 metabolic process	modification-dependent macromolecule catabolic process (GO:0043632)	16	-1.00	-3.30	4.00
		ubiquitin-dependent protein catabolic process (GO:0006511)	15	-0.80	-3.00	3.80
		modification-dependent protein catabolic process (GO:0019941)	15	-0.77	-2.90	3.70
		purine nucleoside triphosphate biosynthetic process (GO:0009145)	4	-0.77	-2.90	5.20
		purine ribonucleoside triphosphate biosynthetic process (GO:0009206)	4	-0.77	-2.90	5.20
AB_orange	GO:0048519 -ve reg. of biological process	-ve reg. of organelle organization (GO:0010639)	4	-0.42	-2.10	3.70
AB_skyblue	GO:0009987 cellular process	centrosome cycle (GO:0007098)	3	-0.71	-2.80	5.80
		microtubule organizing centre organization (GO:0031023)	3	-0.54	-2.50	4.90
GB_bisque4	GO:0009987 cellular process	cell cycle process (GO:0022402)	43	-2.80	-6.20	5.60
		cell division (GO:0051301)	31	-2.70	-5.90	5.60
		nuclear division (GO:0000280)	18	-2.70	-5.80	6.00
		nuclear chromosome segregation (GO:0098813)	15	-2.70	-5.70	6.10
		cell cycle (GO:0007049)	55	-2.60	-5.50	5.10
GB_brown4	GO:0009987 cellular process	microtubule cytoskeleton organization (GO:0000226)	11	-3.40	-7.30	8.60
		microtubule-based process (GO:0007017)	13	-3.40	-7.10	7.80
		microtubule bundle formation (GO:0001578)	5	-2.70	-5.80	11.00
		cilium or flagellum-dependent cell motility (GO:0001539)	4	-2.60	-5.50	12.00
		cilium-dependent cell motility (GO:0060285)	4	-2.60	-5.50	12.00
GB_darkgreen	GO:0048519 -ve reg. of biological process	-ve reg. of protein catabolic process (GO:0042177)	6	-1.20	-3.20	4.60
		-ve reg. of proteasomal ubiquitin-dependent protein catabolic process (GO:0032435)	3	-0.55	-2.20	3.90
		-ve reg. of ubiquitin-dependent protein catabolic process (GO:2000059)	3	-0.55	-2.20	3.90
		-ve reg. of proteolysis involved in protein catabolic process (GO:1903051)	3	-0.55	-2.20	3.90
		-ve reg. of proteasomal protein catabolic process (GO:1901799)	3	-0.55	-2.20	3.90
GB_darkgrey	GO:0008152 metabolic process	RNA processing (GO:0006396)	100	-5.90	-9.90	7.00
		mRNA metabolic process (GO:0016071)	65	-4.80	-8.20	6.40
		RNA splicing (GO:0008380)	47	-3.60	-6.70	5.80
		mitochondrial ATP synthesis coupled electron transport (GO:0042775)	17	-3.50	-6.60	6.40
		ATP synthesis coupled electron transport (GO:0042773)	17	-3.30	-6.40	6.30
GB_darkorange	GO:0032501 multicellular organismal process	nervous system process (GO:0050877)	9	-1.40	-3.60	4.80
		sensory perception (GO:0007600)	5	-0.76	-2.60	4.10
GB_floralwhite	GO:0008152 metabolic process	translational elongation (GO:0006414)	8	-2.70	-5.90	8.00
		amide biosynthetic process (GO:0043604)	18	-1.40	-3.50	4.20
		amide metabolic process (GO:0043603)	21	-1.30	-3.40	4.00
		translation (GO:0006412)	14	-0.91	-2.80	3.60
		peptide biosynthetic process (GO:0043043)	14	-0.84	-2.70	3.50
GB_ivory	GO:0008152 metabolic process	DNA metabolic process (GO:0006259)	8	-0.59	-2.70	3.80
		steroid metabolic process (GO:0008202)	3	-0.16	-2.00	3.80
GB_magenta	GO:0032502 developmental process	striated muscle cell differentiation (GO:0051146)	24	-12.00	-15.00	13.00
		muscle cell differentiation (GO:0042692)	26	-11.00	-15.00	12.00
		muscle cell development (GO:0055001)	24	-11.00	-15.00	12.00
		muscle structure development (GO:0061061)	30	-11.00	-14.00	11.00
		muscle organ development (GO:0007517)	20	-8.70	-12.00	11.00
GB_mediumpurple3	GO:0051179 localization	mitochondrial outer membrane permeabilization (GO:0097345)	4	-1.60	-4.00	6.70
		mitochondrial outer membrane permeabilization involved in programmed cell death (GO:1902686)	4	-1.60	-4.00	6.70
		+ve reg. of mitochondrial membrane permeability involved in apoptotic process (GO:1902110)	4	-1.60	-4.00	6.70
		+ve reg. of mitochondrial membrane permeability (GO:0035794)	4	-1.60	-4.00	6.70
		reg. of mitochondrial membrane permeability involved in apoptotic process (GO:1902108)	4	-1.40	-3.60	6.10
GB_salmon4	GO:0009987 cellular process	extracellular structure organization (GO:0043062)	7	-3.60	-6.20	9.10
		extracellular matrix organization (GO:0030198)	7	-3.60	-6.20	9.10
		external encapsulating structure organization (GO:0045229)	7	-3.60	-6.20	9.10
		collagen fibril organization (GO:0030199)	3	-1.60	-4.00	9.40
GB_skyblue2	GO:0022414 reproductive process	binding of sperm to zona pellucida (GO:0007339)	3	-2.80	-6.20	20.00
		sperm-egg recognition (GO:0035036)	3	-2.80	-6.20	20.00
		reg. of fertilization (GO:0080154)	3	-2.40	-5.80	18.00
		reg. of reproductive process (GO:2000241)	4	-2.40	-5.70	13.00
		single fertilization (GO:0007338)	3	-1.30	-4.10	9.90
GB_thistle2	GO:0032501 multicellular organismal process	myelination (GO:0042552)	5	-2.70	-5.50	10.00
		axon ensheathment (GO:0008366)	5	-2.70	-5.50	9.90

		ensheathment of neurons (GO:0007272)	5	-2.70	-5.50	9.90
		central nervous system development (GO:0007417)	9	-0.73	-2.80	3.80
		glial cell development (GO:0021782)	3	-0.45	-2.20	4.30
HB_blue	GO:0008152 metabolic process	translation (GO:0006412)	97	-19.00	-22.00	11.00
		peptide biosynthetic process (GO:0043043)	98	-19.00	-22.00	11.00
		peptide metabolic process (GO:0006518)	102	-17.00	-20.00	11.00
		amide biosynthetic process (GO:0043604)	103	-14.00	-17.00	9.60
		amide metabolic process (GO:0043603)	121	-13.00	-16.00	9.20
HB_darkgreen	GO:0008152 metabolic process	peptidyl-arginine modification (GO:0018195)	5	-1.00	-3.40	5.10
		macromolecule catabolic process (GO:0009057)	54	-0.96	-3.30	3.60
		one-carbon metabolic process (GO:0006730)	7	-0.96	-3.20	4.50
		proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161)	26	-0.96	-3.20	3.70
		proteasomal protein catabolic process (GO:0010498)	27	-0.94	-3.20	3.60
HB_floralwhite	GO:0009987 cellular process	protein-DNA complex organization (GO:0071824)	18	-0.92	-3.10	3.80
		chromatin organization (GO:0006325)	15	-0.52	-2.50	3.20
		non-motile cilium assembly (GO:1905515)	4	-0.46	-2.40	4.10
		chromatin remodelling (GO:0006338)	10	-0.36	-2.10	3.00
HB_pink	GO:0051179 localization	transcytosis (GO:0045056)	4	-1.50	-3.80	5.70
		vesicle uncoating (GO:0072319)	3	-0.50	-2.30	4.10
		clathrin coat disassembly (GO:0072318)	3	-0.50	-2.30	4.10
		synaptic vesicle uncoating (GO:0016191)	3	-0.50	-2.30	4.10
		establishment of organelle localization (GO:0051656)	24	-0.48	-2.30	2.90
HB_skyblue	GO:0008152 metabolic process	sterol biosynthetic process (GO:0016126)	12	-14.00	-17.00	22.00
		secondary alcohol biosynthetic process (GO:1902653)	11	-14.00	-17.00	22.00
		cholesterol biosynthetic process (GO:0006695)	11	-14.00	-17.00	22.00
		cholesterol metabolic process (GO:0008203)	13	-12.00	-15.00	18.00
		steroid biosynthetic process (GO:0006694)	13	-12.00	-15.00	17.00
HB_thistle1	GO:0051179 localization	protein targeting to vacuole (GO:0006623)	3	-1.70	-4.20	10.00
		establishment of protein localization to vacuole (GO:0072666)	3	-0.96	-3.20	7.00
		vacuolar transport (GO:0007034)	4	-0.92	-3.10	5.60
		protein localization to vacuole (GO:0072665)	3	-0.85	-3.00	6.30
		protein targeting (GO:0006605)	4	-0.46	-2.40	4.20
AL_magenta	GO:0050896 response to stimulus	response to lipid (GO:0033993)	7	0.00	-3.20	4.60
		response to external biotic stimulus (GO:0043207)	12	0.00	-3.10	4.00
		response to biotic stimulus (GO:0009607)	12	0.00	-3.10	4.00
		response to organic substance (GO:0010033)	20	0.00	-2.70	3.40
		response to oxygen-containing compound (GO:1901700)	11	0.00	-2.50	3.40
GL_darkmagenta	GO:0050896 response to stimulus	response to steroid hormone (GO:0048545)	3	-0.41	-3.30	7.10
		response to organic cyclic compound (GO:0014070)	3	-0.04	-2.30	4.50
GL_darkslateblue	GO:0048519 -ve reg. of biological process	-ve reg. of neuron apoptotic process (GO:0043524)	6	-0.71	-4.50	6.50
		-ve reg. of apoptotic process (GO:0043066)	15	-0.71	-4.20	4.80
		-ve reg. of programmed cell death (GO:0043069)	15	-0.69	-4.10	4.70
		-ve reg. of cell development (GO:0010721)	6	-0.01	-2.10	3.30
GL_lightsteelblue	GO:0009987 cellular process	cell-matrix adhesion (GO:0007160)	3	-1.50	-3.70	8.60
		cell-substrate adhesion (GO:0031589)	3	-1.30	-3.40	7.50
GL_mediumorchid	GO:0008152 metabolic process	reg. of cellular catabolic process (GO:0031329)	8	-1.00	-3.10	4.30
		reg. of catabolic process (GO:0009894)	9	-0.79	-2.70	3.80
		reg. of mRNA metabolic process (GO:1903311)	6	-0.66	-2.50	3.80
		lipid catabolic process (GO:0016042)	6	-0.43	-2.10	3.30
		reg. of RNA stability (GO:0043487)	4	-0.41	-2.10	3.60
GL_yellow4	GO:0050789 reg. of biological process	reg. of microtubule cytoskeleton organization (GO:0070507)	3	-0.47	-3.50	7.80
		reg. of microtubule-based process (GO:0032886)	3	-0.40	-3.00	6.40
HL_darkolivegreen	GO:0009987 cellular process	protein-DNA complex organization (GO:0071824)	8	-1.00	-2.90	4.10
		chromatin remodelling (GO:0006338)	5	-0.55	-2.30	3.70
HL_greenyellow	GO:0008152 metabolic process	oxidative phosphorylation (GO:0006119)	14	-8.80	-12.00	12.00
		proton motive force-driven ATP synthesis (GO:0015986)	9	-8.20	-11.00	15.00
		aerobic respiration (GO:0009060)	15	-7.50	-10.00	11.00
		cellular respiration (GO:0045333)	16	-7.30	-10.00	10.00
		ATP biosynthetic process (GO:0006754)	9	-7.30	-10.00	13.00
HL_lightcyan	GO:0051179 localization	intra-Golgi vesicle-mediated transport (GO:0006891)	17	-5.40	-7.90	6.30
		Golgi vesicle transport (GO:0048193)	62	-4.50	-6.80	5.50
		retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum (GO:0006890)	20	-4.20	-6.60	5.70
		endoplasmic reticulum to Golgi vesicle-mediated transport (GO:0006888)	34	-3.70	-6.00	5.20
		protein targeting to ER (GO:0045047)	16	-3.00	-5.20	5.00
HL_violet	GO:0008152 metabolic process	RNA processing (GO:0006396)	46	-2.40	-6.50	5.80
		reg. of mRNA metabolic process (GO:1903311)	17	-1.80	-5.30	5.70
		mRNA metabolic process (GO:0016071)	30	-1.80	-5.20	5.20
		mRNA processing (GO:0006397)	25	-1.30	-4.60	4.80
		RNA splicing (GO:0008380)	22	-1.10	-4.40	4.80

3.4 Discussion

In this experiment I examined divergence in gene expression and gene expression plasticity between sticklebacks from different thermal habitats. Robust divergence in brain gene expression was found in a small number of genes that were divergent between geothermal and ambient sticklebacks at both rearing temperatures. While these genetic differences might be non-adaptive results of genetic drift, there are several arguments in favour of an adaptive hypothesis. I also found evidence of divergence in plastic responses to temperature in the brains of geothermal and ambient sticklebacks. Gene expression in the brains of geothermal fish was more plastic than in ambient fish. I also assessed signs of disrupted gene expression and gene expression plasticity in hybrid fish. Gene expression in the hybrid brain was very different from pure-strains, suggesting widespread disruption. I also found evidence that hybrids show a plastic response that is greatly disrupted when compared to either pure-strain type, potentially indicating another area in which hybrid fitness may be impacted in the wild. Finally, I investigated the regulatory mechanisms behind patterns of geothermal and ambient gene expression. I found little evidence of prominent additive variation. I found a large amount of transgressive expression in hybrids, and no evidence of significant levels of cis-regulatory elements.

3.4.1 Divergence between geothermal and ambient sticklebacks

We unexpectedly found only a small number of robust divergent genes in the brain, potentially a sign of limited divergence between geothermal and ambient sticklebacks due to the young age of the habitat. The geothermal habitat used in this experiment was formed approximately 70 years ago by the output of geothermal heating systems in nearby residential buildings. While the evolutionary process has traditionally been thought of as a slow, prolonged process, examples of rapid evolutionary divergence are not rare (Hairston et al., 2005; Kopp and Matuszewski, 2014; Messer et al., 2016), including in an example of fish adapting to warmed habitats (Dayan et al., 2019). The geothermal-ambient pair used in my experiment is allopatric, and so no gene flow is expected to have occurred between the geothermal and ambient fish, encouraging divergence. As such, the age of this habitat is not necessarily restrictive to evolutionary divergence. Furthermore, heritable divergence has been found in morphology in this geothermal-ambient pair, suggesting that

evolutionary divergence has occurred in this trait (see the Allopatric 1 pair in Pilakouta et al., 2023). As such, I expected to find a greater amount of robust divergence between geothermal and ambient sticklebacks in this population. However, I did find evidence of divergence in the co-expression of genes, as several modules in the brains and livers of geothermal fish were not preserved in ambient fish. This result suggests that the ways in which genes in these modules interact with other genes may have diverged in geothermal fish. Divergence between geothermal and ambient habitats may therefore involve widespread, subtle changes in gene expression within these divergent modules.

The genes that did exhibit robust divergence in gene expression may be a result of genetic divergence or transgenerational effects, potentially suggesting an important role for these genes in thermal adaptation. The genes trpc4a, tmem59l, wdr91 and dpysl2b were downregulated in the brains of geothermal fish. These genes are implicated in processes involving brain cell function and development (Liu et al., 2017; Von Niederhäusern et al., 2013; Suzuki et al., 2022; Zheng et al., 2017). The downregulation of these genes may indicate a reduction in brain cell function, potentially as an adaptation to the geothermal habitat. Fish acclimated to higher temperatures tend to show affected brain functions. This can take the form of altered synaptic transmission (Szabo et al., 2008), reduced mitochondrial performance in brain cells (Chung et al., 2017) and an impairment to normal cognitive abilities and behaviors (Pilakouta et al., 2022; Szabo et al., 2008; Závorka et al., 2020).

One way in which reduced brain cell function and development may benefit fish in a geothermal habitat is by reducing the energy demands of the brain. Fish colonizing a warmed habitat experience an elevated metabolic rate, which in turn increases energy expenditure and the energy required to survive (Fry, 1967). Geothermal sticklebacks across multiple geothermal-ambient pairs have been found to exhibit a reduced metabolic rate when compared to ambient sticklebacks at a common temperature (Pilakouta et al., 2020). However, the exact mechanisms behind this reduced metabolic rate are not known. The brain is a metabolically costly organ, requiring much more energy than other organs when at rest (Heldstab et al., 2022; Mink et al., 1981). At the higher temperatures of the geothermal habitat the metabolic burden of the brain may present a particular challenge. As such, adaptations to reduce this metabolic

burden may be effective in improving fitness. Indeed, the size of the brain in vertebrates has been found to be constrained by temperature (Gillooly and McCoy, 2014) and seasonal food availability (Luo et al., 2017; Weisbecker et al., 2015; van Woerden et al., 2012; Van Woerden et al., 2014). Additionally, the resources an organism invests in the brain depends on the amount of energy allocated to other bodily functions (Kotrschal et al., 2013; Tsuboi et al., 2014). If adaptation to the geothermal habitat involves investment in alternate organs or tissues, then this may come at a cost to investment in the brain. The brains of fish in a warmed habitat are also potentially affected by oxygen limitation. The concentration of dissolved oxygen in water decreases with increasing temperature, occurring alongside an increase in the oxygen demands of the metabolism in fish (McBryan et al., 2013; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2005). This can result in a severe mismatch between oxygen demand and delivery, presenting a major restriction to activity and potentially threatening survival (Andreassen et al., 2022; Pörtner and Farrell, 2008). These effects can be particularly severe in the brain, with impaired brain function caused by insufficient oxygen availability during warming (Andreassen et al., 2022). Reduced investment in the brain is expected in environments with low oxygen availability (Chapman and Hulen, 2001; Crispo and Chapman, 2010; Safi et al., 2005). Acclimation to increased temperatures through an increase in aerobic scope has been found to come at a cost to cognitive abilities in fish, even despite observed increases brain volume (Závorka et al., 2020). This suggests that adaptation of the fish brain to warmed habitats is likely to be complex, involving a variety of trade-offs. Further investigation into the anatomy of the brains of geothermal sticklebacks may reveal whether particular regions of the brain are affected. Reduced neuron development and function focused in a specific region of the brain would indicate that the functions of that region are likely to be impaired. Additionally, the consequences of this divergence on stickleback behavior and cognitive ability may reveal trade-offs, where geothermal sticklebacks may be less capable at certain activities when compared to ambient sticklebacks.

3.4.1.1 Gene expression plasticity in geothermal and ambient sticklebacks

The greater plasticity of the geothermal brain is unexpected, as adaptation to a novel habitat is expected to result in a reduction in plasticity over time, as initially plastic responses are refined and canalized into genetic

divergence (Levis and Pfennig, 2016, 2019c). However, in this experiment, the greater number of plastic genes in geothermal sticklebacks and the little evidence I found of genetic divergence, suggests that adaptation to the geothermal habitat largely consists of plastic responses. Furthermore, several brain and livers modules in both geothermal and ambient fish were plastic, while the corresponding modules in the alternate pure-strain network were not, suggesting that divergent plasticity involves coordinated changes to interactions between large sets of genes.

As the geothermal habitat was colonized by fish from the ambient habitat, the ambient plastic response is expected to represent the ancestral plastic response. The unique plastic responses found in both geothermal and ambient fish in this experiment therefore suggests both a loss of ancestral plasticity and a gain of new plasticity in geothermal fish. Ancestral plasticity may be lost during a refinement of the plastic response to better suit long term survival in the geothermal habitat (Levis and Pfennig, 2019c). Refinement may have occurred through selection against non-adaptive plasticity (Ghalambor et al., 2015). The unique geothermal brain plastic response was found to involve a large number of genes relating to metabolic processes. This divergence in the plasticity of metabolic processes is potentially related to the divergence in metabolic rate found to be consistent across geothermal sticklebacks (Pilakouta et al., 2020). The geothermal brain was found to be plastic in a wide array of metabolic processes, suggesting a plastic response likely to have a widespread biological effect. This result further strengthens the argument that the metabolism of fish is particularly important in thermal adaptation.

3.4.2 Differential expression and plasticity between hybrid and pure strain

Selection against inter-population hybrids can contribute to the formation of reproductive isolation between diverging populations (Kulmuni et al., 2020). Divergent populations are expected to be locally adapted and hybrids inheriting intermediate or transgressive phenotypes are likely to face fitness consequences (Chhina et al., 2022; Gow et al., 2007; Hartman et al., 2013; Rice and McQuillan, 2018; Thompson et al., 2021). As pure-strain gene expression is expected to be adapted to their native habitats (Ayroles et al., 2009; Brauer et al., 2017), hybrid expression differing from this is likely to be maladaptive in the wild. Indeed, hybrid mis-expression has been found to accompany severe fitness

consequences (Barreto et al., 2015; Ellison and Burton, 2008; Ortíz-Barrientos et al., 2007). In this experiment we found that hybrid gene expression differed greatly from both geothermal and ambient fish in genes and gene modules covering a wide range of ontologies. As gene expression is expected to correlate with biology (Dalziel et al., 2018), my results suggest that these hybrid fish may experience alterations in the functioning of a large number of biological processes. Metabolic processes were particularly affected in hybrids, which may be especially deleterious in the geothermal habitat considering the likely importance of metabolism in geothermal adaptation (Pilakouta et al., 2020). Furthermore, the hybrid plastic response was more extensive than either pure-strain response, with a larger number of genes involved and a wider distribution of affected gene ontologies. This result fits with previous work investigating hybrid plastic responses, where the hybrid plastic response is broader than the parental response (Bernatowicz et al., 2021; Gallego-Tévar et al., 2018). This is likely to be detrimental in the wild, where a fine-tuned response to the correct stimulus is optimal. Overall, my results suggest that these hybrid fish would be likely to exhibit poor fitness in the wild indicating a potential reproductive barrier between geothermal and ambient fish, despite the small amount of robust divergence found between these fish.

Additionally, we found a large number of transgressively expressed genes in hybrid sticklebacks. Transgressive phenotypes are often maladaptive (Pauers et al., 2022). Alternatively, transgressive phenotypes rapidly generate novel trait values, potentially aiding evolution (Dittrich-Reed and Fitzpatrick, 2013; Gallego-Tévar et al., 2019). This phenomenon is thought to have contributed to the rapid divergence of cichlids (Holzman and Hulsey, 2017), with evidence for frequent instances of gene flow between diverging lineages (Malinsky et al., 2018). Transgressive gene expression has been found to underlie improved growth in hybrids of channel (*Ictalurus punctatus*) and blue (*Ictalurus furcatus*) catfish (Wang et al., 2022). As such, the effects of widespread transgressive gene expression in geothermal-ambient hybrids may be complex. Further study of fitness metrics of these hybrids would provide great insight into the potential impacts of gene flow between thermal habitats.

3.4.2.1 Mechanics underlying divergence.

We found little evidence that additive variation contributed to divergence between geothermal and ambient sticklebacks, a result which would classically be interpreted as suggesting little potential for evolution (Falconer and Mackay, 1996). This result is unexpected, as transcriptional variation is largely expected to be additive (Gilad et al., 2008; Kim and Gibson, 2010). However, widespread non-additive inheritance in gene expression has previously been found in other vertebrates (Debes et al., 2012), including sticklebacks (Leder et al., 2014). Approximately 10% of gene expression variation was found to be parentally dominant in geothermal-ambient hybrid sticklebacks. The mechanisms behind the evolution of dominance have been hotly debated (Bagheri, 2006). One hypothesis suggests that dominance is likely to evolve in metabolic pathways, as a result of selection for robustness to genetic and environmental perturbations (Bagheri and Wagner, 2004; Bourguet, 1999). As geothermal and ambient sticklebacks have been found to exhibit divergent metabolic rates (Pilakouta et al., 2020), and divergent plasticity in metabolic processes in this experiment, the gene mechanics behind these metabolic processes may also be diverging. Metabolic processes were consistently involved in dominance of by parental types and at both rearing temperatures. A limitation of this experimental design is that hybrid crosses were generated from ambient sourced males and geothermal sourced females, with no reciprocal cross. A fully reciprocal cross would allow for a more detailed examination of geothermal and ambient dominance, as well as the degree to which hybrid expression is affected by paternal and maternal effects.

A very large portion, approximately 30%, of the expressed genes in the hybrid brain were transgressively expressed, suggesting a large amount of disruption to regulatory mechanisms. This result suggests that geothermal and ambient fish have diverged in the regulatory mechanisms that affect gene expression. However, I found little evidence of cis-regulatory divergence in geothermal and ambient sticklebacks. Divergence in cis-regulatory processes is expected to underlie evolutionary divergence in gene expression more often than trans-regulatory changes (Signor and Nuzhdin, 2018; Verta and Jones, 2019; Wittkopp et al., 2004; Zhong et al., 2019). Selection acts more effectively on cis-regulatory variation as it is more strongly associated with additive loci (Lemos et al., 2008). In this experiment we found little evidence of

contributions from either additive variation, or cis-regulatory elements to divergence in geothermal and ambient sticklebacks.

3.4.3 Conclusions

Our data suggests that the brain is a key organ in thermal adaptation, as gene expression in this organ was found to be divergent between geothermal and ambient fish, and highly plastic in response to temperature, with geothermal fish showing a divergent plastic response to ambient fish. Additionally, gene expression in the brains of hybrids was highly disrupted and involved a large number of transgressively expressed genes, potentially indicating the formation of a reproductive barrier between geothermal and ambient fish. Divergence between geothermal and ambient fish, and disruption in hybrids is primarily in metabolism related gene ontologies, suggesting an important role for metabolism in thermal adaptation. I also found evidence for divergence in neuron development and functioning, suggesting potential routes for future research in this study system. However, I also found that the divergence between geothermal and ambient fish was largely plastic, and I found little evidence for additive variation in gene expression, or cis-regulatory divergence, suggesting little evolutionary divergence between geothermal and ambient sticklebacks. This may be due to the young age of the study system, which is estimated to be around 70 years old. This result may also suggest that plasticity may have limited the effects of selection on the genetic makeup of geothermal and ambient sticklebacks. Furthermore, the divergence in plastic response and the loss of a coordinated plastic response in hybrids suggests that phenotypic plasticity may be under selection. This result also highlights the importance of the plastic response in the colonization of novel habitats, as the plastic response of geothermal fish has diverged significantly from that of ambient fish.

4 Chapter 4: The consistency of divergence and plasticity of gene expression across multiple populations of geothermal-ambient stickleback pairs

4.1 Introduction

4.1.1 The threat of climate change

Increasing environmental temperatures driven by climate change presents a threat to biodiversity (France et al., 2013; IPCC, 2021). Wild populations are likely to face strong selective pressures and, if range shifts are not possible, will need to adapt or change to persist (Parsons, 2021). Temperature affects many aspects of biology at a fundamental level and with wide-ranging ramifications, from the molecular to the ecological (Brown et al., 2004). As temperature affects such a wide array of biological processes, there are many potential routes for adaptation to a warmed habitat, so predicting whether or not a population will be able to adapt to a warming environment is often challenging. The concept of evolvability describes the ability of a species to adaptively evolve (Campbell et al., 2017; Feiner et al., 2021; Parsons, 2021). Evolvability depends on not only the sheer amount of genetic variation, but also the adaptive potential of this variation and the interactions between genotype, phenotype and environment (Campbell et al., 2017). Phenotypic plasticity, the ability for a single genotype, or developmental system, to produce multiple phenotypes dependent on environmental cues (Bradshaw, 1965; Parsons, 2021), is an important part of the concept of evolvability. Phenotypic plasticity can improve the evolvability of a population by allowing for a rapid, beneficial response to novel conditions that permits persistence, essentially "buying time" for evolutionary change to occur. In the case of increasing environmental temperatures, many organisms are able to acclimate to temperatures somewhat out of their optimal range when allowed the time to do so (Lagerspetz, 2006). However, there are limits to acclimation, and so further adaptation may be required. Under the hypothesis of "plasticity-led evolution" these initial adaptive, but suboptimal, plastic responses to novel conditions may then be refined and canalised through genetic changes over time (Levis and Pfennig, 2016, 2019b). The evolutionary process can also be benefited by plastic response through the revealing of cryptic genetic variation to selection (Parsons et al.,

2020). Wild populations that show plastic responses to increased temperature are therefore expected to be less vulnerable to climate change.

4.1.1.1 Temperature and fish

Fishes are likely to be especially threatened by increasing temperatures due to their ectothermic nature (Donelson et al., 2012; Fry, 1967). This is especially true for freshwater fishes, as freshwater habitats provide limited capacity for migration (Dudgeon et al., 2006), many freshwater fishes will likely need to adapt and change in order to persist under higher temperatures (Crozier and Hutchings, 2014). The metabolic rate of fishes is greatly influenced by the environmental temperature (Fry, 1967). Higher temperatures induce a faster standard metabolic rate, increasing the costs of basic maintenance for the organism and reducing the scope for activity and growth. At extreme temperatures this metabolic scope may be reduced enough that oxygen availability and transport becomes a major restriction and survival may be threatened (Fry, 1967; Pörtner and Farrell, 2008). Even small temperature-induced increases in standard metabolic rate can impose fitness consequences. As more energy is required for basic maintenance and additional activity, calorific requirements are increased and fishes in warmed water must increase food intake to compensate (Neubauer and Andersen, 2019; Volkoff and Rønnestad, 2020). Fishes in natural habitats with increased temperatures may compensate for increased calorific requirements through altered prey selection (O'Gorman et al., 2016). Altered prey selection may result in a divergence of foraging behaviour and anatomy. Indeed, morphological divergence consistent with a shift towards a more benthic lifestyle has been observed in fishes inhabiting warmed habitats (Lema et al., 2019; Pilakouta et al., 2023; Rocamontes-Morales et al., 2021; Rowiński et al., 2015). The effects of temperature on fish also extends to the immune system. Optimal immune system performance typically occurs at temperatures intermediate to the thermal range of a species (Abram et al., 2017; Bowden, 2008; Makrinos and Bowden, 2016; Scharsack and Franke, 2022). Fish exposed to temperatures at the higher end of their typical range tend to exhibit an increase in antibody related functions (Makrinos and Bowden, 2016). However, sufficiently high temperatures can suppress innate and acquired immune system functioning, increasing the risk of infection and potentially further reducing fitness at high temperatures (Dittmar et al., 2014; Scharsack and Franke, 2022). A compromised immune system may

be particularly detrimental in cases where the parasite or pathogen benefits from the increased temperature (Franke et al., 2017, 2019; Lõhmus and Björklund, 2015; Macnab and Barber, 2012; Scharsack and Franke, 2022).

The potential impacts of climate change driven increases in temperature on freshwater fishes are likely to be widespread and affecting complex traits such as metabolism and immunity. As such, assessing the potential for adaptation to these impacts can be difficult. Whole transcriptome sequencing is a valuable tool in this research, as gene expression can provide an objective overview of a vast number of biological traits simultaneously (Brauer et al., 2017; Houle et al., 2010). Comparative studies of the gene expression of populations from different thermal habitats can be used to reveal diverging traits that may contribute to thermal adaptation (Brauer et al., 2017). For example, the assessment of differential expression (DE) between fish from warmed and ambient habitats has revealed divergence in genes relating to respiration (Garvin et al., 2015) and heat shock proteins (Mahanty et al., 2017; Oksala et al., 2014). Additionally, gene expression can reveal phenotypic plasticity at the molecular level, and elucidate the mechanisms and traits involved in plastic responses to thermal stress (Alvarez et al., 2015; Drown et al., 2022; Logan and Buckley, 2015; Metzger and Schulte, 2018).

4.1.2 Study design.

Understanding the potential routes of adaptation and adaptive plastic responses to increased environmental temperatures is key to assessing the ability of freshwater fishes populations to persist in the face of climate change (Parsons, 2021). Geothermal habitats are a valuable tool for such research (Woodward et al., 2010). The repeated colonization of geothermal habitats from nearby ambient habitats provides multiple examples of thermal adaptation. Furthermore, confounding factors such as photoperiod, geology, and vegetation, are very similar across geothermal and ambient habitat pairs due to their close proximity to each other. Geothermal activity is particularly prevalent in Iceland, and many geothermally warmed habitats can be found in close proximity to ambient temperature habitats. Several of these geothermal-ambient habitat pairs have been found to be inhabited by three-spined sticklebacks (*Gasterosteus aculeatus*) (Millet et al., 2013; Pilakouta et al., 2020, 2023). This study system presents a unique opportunity to test for consistent patterns in

geothermal-ambient fish divergence and the importance of plasticity for such divergence. Indeed, stickleback in these systems have been found to be consistently divergent in metabolic rate, with stickleback in geothermally warmed exhibiting a lower standard metabolic rate when kept at a common temperature with stickleback from ambient systems (Pilakouta et al., 2020). Furthermore, heritable and consistent divergence in morphology further suggests that geothermal sticklebacks have adapted to the warmed thermal habitat in a range of traits (Pilakouta et al., 2023).

In the experiment presented here I aimed to assess gene expression divergence and plasticity in geothermal and ambient stickleback pairs across three population pairs. I assessed geothermal and ambient sticklebacks for evidence of divergence in gene expression that is no affected by rearing temperature, in other words, robust divergence that may indicate genetic divergence. I further assessed whether this robust divergence was found in all three population pairs, as a consistency in divergence would support the hypothesis that these divergent genes are a part of an adaptive response to thermal habitat. Additionally, I tested for evidence of adaptive plasticity by assessing for divergent plastic responses between geothermal and ambient sticklebacks. Specifically, I assessed for genes that are consistently plastic in geothermal, but not ambient sticklebacks and vice versa. Genes that are consistently uniquely plastic in either geothermal or ambient fish are evidence of divergent adaptive plasticity in geothermal fish.

The key questions of the experiment were as follows: 1. Is there a consistent pattern of divergence in gene expression between geothermal and ambient sticklebacks across population pairs? and 2. Is there divergence in the geothermal and ambient plastic response to rearing temperature that is consistent across geothermal-ambient stickleback pairs, suggesting adaptive plasticity. I predict that I will find evidence of consistent patterns of divergence in gene expression across the three population pairs. I predict that divergence and plasticity will be found in genes relevant to thermal adaptation, particularly those relating to metabolic pathways (Fry, 1967; Goldspink, 1995; Kim et al., 2017; Oomen and Hutchings, 2017; Pilakouta et al., 2020; Ryu et al., 2018).

4.2 Methods

4.2.1 Creation of F1 generation.

Stickleback population pairs from two sympatric and one allopatric geothermal-ambient habitat pair from Northern Iceland were used in this experiment: Áshildarholtsvatn (ASHN, sympatric), Mývatn (MYV, sympatric) and an unnamed pair of water bodies near Sauðárkrókur (SKR, allopatric) (Table 4-1). The geothermal habitat at the MYV site is approximately 2,300 years old and is heated by natural geothermal activity (Einarsson et al., 2004). The geothermal habitats at the ASHN and SKR sites are both significantly younger, formed within the past 70 years by the excess hot water run-off from the geothermal heating systems of nearby residences.

Table 4-1 Site and habitat data concerning the three populations used in this experiment.

Population pair name	Type	Thermal habitat	GPS coordinates for sampling sites	Distance between geothermal and ambient habitats (km)	Age of habitat (estimate in years)	Summer temp. (°C)	Winter temp. (°C)	Dissolved Oxygen (mg/l)
Mývatn (MYV)	Sympatric	Geo.	65.633991, -16.923241	3.18	~2,300	22.8	22.0	6.25
		Amb.	65.630196, -16.991621		~2,300	11.5	1.0	12.55
Áshildarholtsvatn (ASHN)	Sympatric	Geo.	65.72516, -19.600725	0.05	50-70	24.1	12.5	13.4
		Amb.	65.724976, -19.601784		<10,000	12.2	3.4	12.2
Sauðárkrókur, Unnamed (SKR)	Allopatric	Geo.	65.732260, -19.618937	0.02	50-70	25.4	-	7.71
		Amb.	65.732191, -19.618574		<10,000	13.9	-	9.33

Sexually mature adult sticklebacks were gathered from the three geothermal-ambient habitat pairs in 2016 and transported to the University of Glasgow (Figure 4-1). Ambient and geothermal habitats were mimicked in the laboratory system with water temperatures of 12°C and 18°C, respectively. The wild fish were prepared for reproduction with nutrient rich food and changes in light period. Families were created using in vitro fertilisation (IVF) following Barber and Arnott (2000). Male sticklebacks were euthanised using Schedule 1 killing methods, with an overdose of benzocaine solution (12ml per litre) and confirmation of death via cervical dislocation and sperm extracted from the gonadal tissue through maceration in a sterilised container. Gravid female sticklebacks were lightly sedated with benzocaine solution and stripped of eggs into a moistened petri dish through the application of gentle pressure to the abdomen. The macerated gonadal tissue of the male was then added and mixed with a paintbrush. Egg clutches were then covered and left to fertilise for fifteen minutes at room temperature before being split evenly in half after

hardening. Each half went into one of two mimicked thermal habitats (12°C representing the ambient habitat and 18°C representing the geothermal habitat). This experimental design created the following twelve treatment groups: ASHN Ambient at 12°C, ASHN Ambient at 18°C, ASHN Geothermal at 12°C, ASHN Geothermal at 18°C, SKR Ambient at 12°C, SKR Ambient at 18°C, SKR Geothermal at 12°C, SKR Geothermal at 18°C, MYVAT Ambient at 12°C, MYVAT Ambient at 18°C, MYVAT Geothermal at 12°C, and MYVAT Geothermal at 18°C (Figure 4-1). See Table 4-2 for the distribution of samples across treatment groups, tissues and families.

Fertilised egg clutches were reared in mesh baskets submerged in water treated with methylene blue (2.5 µg/ml) until hatching. The system was fed by a recirculated continuous water drip system, with regular water changes and kept well aerated. Photoperiod mimicked the natural photoperiod of Iceland over the year of rearing, including seasonal changes. Daily inspections removed dead or infected eggs. After hatching, juveniles were fed live food (newly hatched brine shrimp (*Artemia salina*), and microworms (*Panagrellus redivivus*)) as well as size appropriate ZM powdered food (ZM100 and ZM200) (ZMsystems, Twyford, UK). At an approximate length of 2cm, juveniles were moved, in their family groups, to 10L tanks at standardized densities of 15-20 individuals. At adulthood the fish were fed trout pellets (Microstart, EWOS Ltd, Surrey, UK).

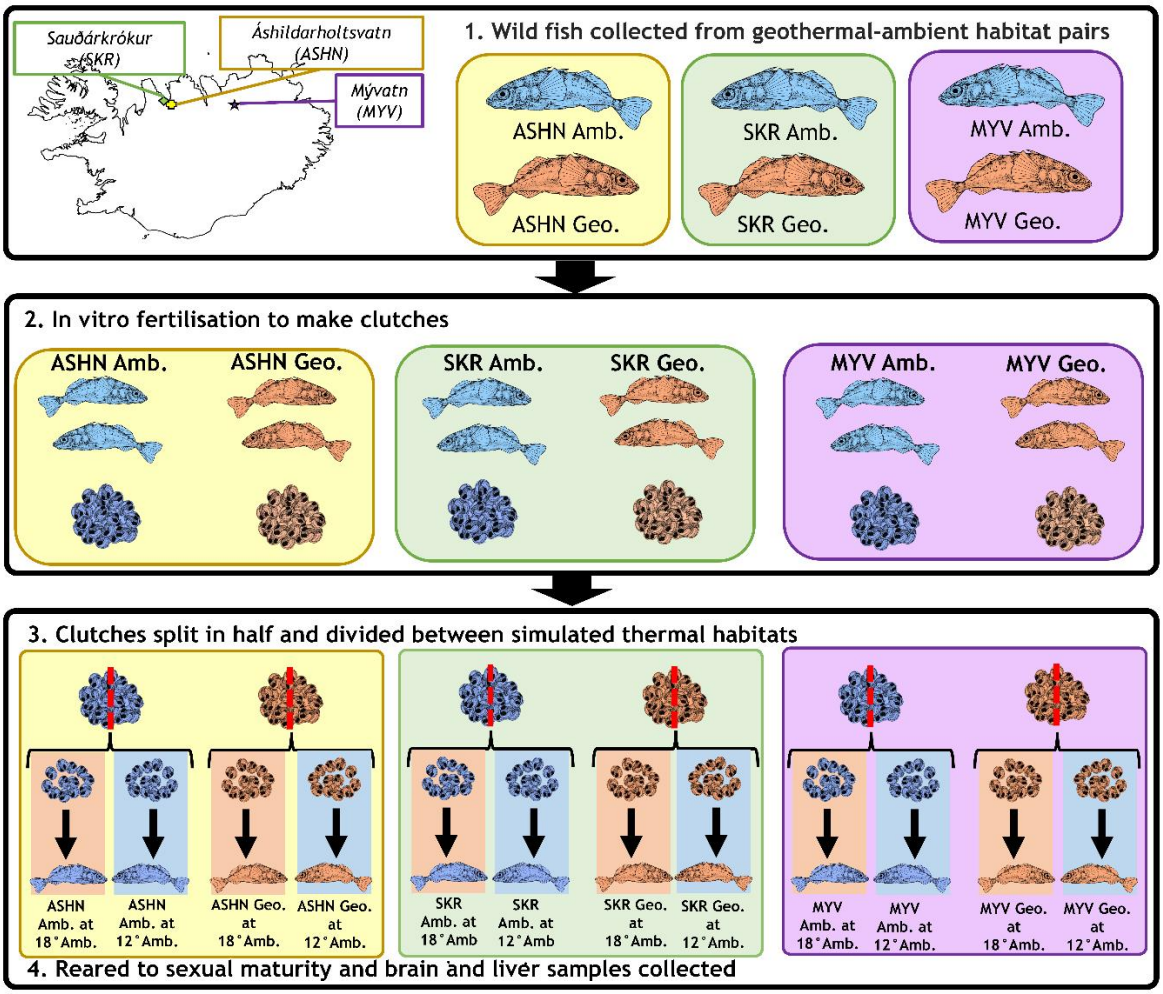


Figure 4-1 Experimental design. 1. Collection of sticklebacks from three geothermal-ambient habitat pairs (ASHN, SKR and MYV) in Northern Iceland. 2. Creation of geothermal and ambient stickleback egg clutches for each population pair and 3. Splitting of clutches between rearing temperature to create twelve treatment groups

Table 4-2 Sample distribution across treatment groups, tissue types and families, including the numbers of samples with more than 10M aligned reads (used in further analysis).

						Samples with >10M aligned reads	
Pop.	**Type**	**Temp. (°C)**	**Tissue**	**Total samples**	**Sample distribution across families: Family code (# samples)**	**Total samples**	**Sample distribution across families Family code (# samples)**
ASH	AMB	12	BRAIN	18	AA1 (3), AA2 (3), AA3 (3), AA4 (9)	12	AA1 (3), AA2 (3), AA3 (2), AA4(4)
			LIVER	18	AA1 (3), AA2 (1), AA3 (3), AA5 (2), AA4 (9)	13	AA1 (3), AA2 (1), AA3 (2), AA5 (2), AA4 (5)
		18	BRAIN	15	AA1 (3), AA3 (1), AA6 (3), AA5 (6), AA4 (2)	14	AA1 (2), AA3 (2), AA6 (3), AA5 (6), 105 (2)
			LIVER	15	AA1 (3), AA3 (1), AA6 (3), AA5 (6), AA4 (2)	14	AA1 (2), AA3 (1), AA6 (3), AA5 (6), AA4 (2)
	GEO	12	BRAIN	21	AG1 (12),AG2 (3), AG3 (3), AG4 (3)	16	AG1 (8), AG2 (2), AG3 (3), AG4 (3)
			LIVER	17	AG1 (8), AG2 (3), AG3 (3), AG4 (3)	17	AG1 (8), AG2 (3), AG3 (3), AG4 (3)
		18	BRAIN	20	AG1 (3), AG2 (3), AG3 (3), AG5 (3), AG6 (6), AG4 (2)	18	AG1 (2), AG2 (3), AG3 (3), AG5 (3), AG6 (5), AG4 (2)
			LIVER	17	AG1 (3), AG2 (3), AG3 (3), AG5 (3), AG6 (3), AG4 (2)	15	AG1 (3), AG2 (2), AG3 (3), AG5 (3), AG6 (2), AG4 (2)
SKR	AMB	12	BRAIN	15	SA1 (3), SA2 (1), SA3 (3), SA4 (5), SA5 (3)	10	SA1 (3), SA2 (1), SA3 (3), SA4 (1), SA5 (2)
			LIVER	15	SA1 (3), SA2 (1), SA3 (3), SA4 (5), SA5 (3)	7	SA1 (3), SA2 (1), SA4 (1), SA5 (2)
		18	BRAIN	15	SA2 (3), SA7 (3), SA6 (3), SA3 (3), SA5 (3)	4	SA7 (1), SA6 (3)
			LIVER	18	SA1 (3), SA2 (3), SA7 (3), SA6 (3), SA3 (3), SA5 (3)	11	SA1 (2), SA2 (1), SA7 (2), SA6 (2), SA3 (2), SA5(2)
	GEO	12	BRAIN	19	SG1 (3), SG2 (9), SG3 (3), SG4 (1), SG5 (3)	6	SG1 (1), SG2 (3), SG3 (1), SG5 (1)
			LIVER	17	SG1 (3), SG2 (5), SG3 (3), SG4 (3), SG5 (3)	11	SG1 (3), SG2 (2), SG3 (1), SG4 (3), SG5 (20
		18	BRAIN	15	SG1 (4), SG3 (5), SG4 (6)	11	SG1 (2), SG3 (3), SG4 (6)
			LIVER	17	SG1 (3), SG2 (3), SG3 (5), SG4 (6)	11	SG2 (2), SG3 (3), SG4 (6)
MYV	AMB	12	BRAIN	18	MA1 (3), MA2 (3), MA3 (3), MA4(3), MA5 (3), MA6 (3)	15	MA1 (3), MA2 (3), MA3 (2), MA4 (3), MA5 (2), MA6 (2)
			LIVER	18	MA1 (3), MA2 (3), MA3 (3), MA4 (3), MA5 (3), MA6 (3)	16	MA1 (3), MA2 (3), MA3 (2), MA4 (2), MA5 (3), MA6 (3)
		18	BRAIN	-	-	-	-
			LIVER	15	MA7 (1), MA1 (3), 026 (3), MA2 (3), MA3 (1), MA5 (1), MA6 (3)	15	MA7 (1), MA1 (3), 026 (3), MA2 (3), MA3 (1), MA5 (1), MA6 (3)
	GEO	12	BRAIN	15	MG1 (3), MG2 (3), MG3 (4), MG4 (5)	12	MG1 (3), MG2 (3), MG3 (3), MG4 (3)
			LIVER	17	MG1 (3), MG5 (2), MG2 (3), MG3 (4), MG4 (5)	17	MG1 (3), MG5 (2), MG2 (3), MG3 (4), MG4 (5)
		18	BRAIN	13	MG1 (6), MG5 (4), MG2 (2), MG3 (1)	12	MG1 (6), MG5 (4), MG2 (2)
			LIVER	16	MG1 (6), MG5 (6), MG2 (2), MG3 (1), MG6 (1)	16	MG1 (6), MG5 (6), MG2 (2), MG3 (1), MG6 (1)

4.2.2 Tissue collection, RNA extraction and sequencing

The F1 generation of lab bred fish were culled at sexual maturity, between October and December of 2018. Fish were euthanised using Schedule 1 killing methods, with an overdose of benzocaine solution (12ml per litre) and confirmation of death via cervical dislocation. Small batches of three fish were sacrificed at a time to minimise the amount of time between euthanasia and tissue preservation. Sticklebacks were weighed, measured for fork length and sexed via identification of testes or ovaries. Sexes were sampled evenly within the treatment groups. Fish were sampled evenly across family groups, with an aim of three fish per family per rearing temperature minimum. Whole brains and livers were collected, cut into pieces smaller than $0.5cm^3$ and stored separately in RNAlater at room temperature over night before freezing at -80°C. Work surfaces, gloves and dissection equipment were cleaned using RNAseZap solution (AM9780, Thermo Fisher Scientific, Massachusetts, US) in between each tissue dissection.

The MYV population pair of fish did not perform well in laboratory conditions, with a higher death rate, particularly in MYV ambient temperature fish reared at 18°C. No external cause for the poor performance of this group was found. The families from each population pair were randomly distributed across tanks in the aquarium, and so the set-up of the aquarium room is unlikely to have caused this increased mortality rate. As such, fewer fish from this group were available for tissue sampling, and the brains of this group could not be sampled for this experiment due to other experimental commitments requiring undamaged heads.

RNA extraction was performed on a total of 384 samples (184 brain and 200 liver samples). RNA was extracted from approximately 20mg of tissue. For liver samples the Qiagen RNeasy standard kit (Qiagen Cat ID: 74104) was used (with the use of 50% ethanol for washes as recommended for fatty tissues). RNA was extracted from brain samples using the Qiagen RNeasy lipid tissue kit for brain tissue (Qiagen Cat ID: 74804), as this was found to provide better results for this tissue than the standard kit.

RNA purity was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, US) and samples with 260/230 or 260/280 ratios

below 1.8 were cleaned using ethanol precipitation and retested. Samples that continued to show poor 260/280 or 260/230 values were replaced with a re-extraction of the same sample, if enough tissue remained, or a suitable replacement (samples from the same treatment group). RNA concentration was determined using a Qubit 4 Fluorometer using a Qubit RNA Broad Range kit (Thermo Fisher Scientific, Massachusetts, US). Tissue samples where the extracted RNA showed low concentrations (below 100ng/ul) were re-extracted. RNA integrity was assessed for all samples using an Agilent 4200 Tapestation machine (Agilent Technologies, California, US). Samples with R.I.N values), a numerical score ranging from 1-10 where a higher value indicates a higher degree of RNA integrity (Sheng et al., 2017), below 7 were re-extracted or replaced and re-tested, 123 samples were re-extracted in this manner and improved RIN values confirmed.

The preparation of cDNA libraries and whole transcriptome sequencing was performed by the CGR at The University of Liverpool. Library preparation was performed using Qiagen's Qiaseq UPXome RNA library kit, using the N6T RT and ODT-T RT primers to generate pools of 24 samples, which were then sequenced using the Novaseq platform (Illumina, California, US).

4.2.3 RNA-seq data analysis

4.2.3.1 Initial data processing and quality control

The obtained Fastq files were first de-multiplexed using the QIAseq sample analysis workflow's *demultiplex* function in the Qiagen CLC workbench (version 23.0.4). Demultiplexed fastq files were trimmed for the presence of Illumina adapter sequences using *Cutadapt* version 1.2.1 (Martin, 2011) and then further trimmed using *Sickle* version 1.200 (Joshi and Fass, 2011). I verified read quality with *FastQC* (Andrews, 2010) and *MultiQC* (Ewels et al., 2016) software. RNA-seq reads were aligned to version 5 of the Threespine stickleback reference genome (Genbank GCA_016920845.1) (Peichel et al., 2020) using the *Hisat2* aligner version 2.2.0 (Kim et al., 2019). Samples with less than 10M aligned reads were omitted from further analysis in this experiment (see Table 4-2). The resulting SAM files, containing the alignment information for each sample, were then sorted and converted to BAM format, a compressed binary version of a SAM file, using *Samtools sort* and indexed using *Samtools index* (Danecek et al., 2021). Read counts were then obtained using *Htsqcount2* (Anders et al., 2015).

4.2.3.2 Differential expression analysis

I performed data analysis in R (v4.3.0) (R Core Team, 2022). Two parallel data analyses pipelines, using *EdgeR* (Robinson et al., 2009) and *LIMMA* (Ritchie et al., 2015) were used to ensure high confidence in genes found to be differentially expressed (DE) in both analyses.

4.2.3.3 Pre-processing of raw counts

Read count data was pre-processed to remove genes with low read counts by filtration for low-expressed genes using *filterbyExpr*. Genes were removed if they had less than ten counts-per-million (CPM) in more than four samples in the brain and seven samples in the liver (the sample size of the smallest group for each dataset) (Chen et al., 2016). To prepare for downstream analysis, read counts were normalized by converting observed library sizes into effective library sizes by calculating scaling factors using *calcNormFactors* with the TMM method (Robinson and Oshlack, 2010).

4.2.3.4 Modelling of count data

To model the effects of fish type and rearing temperature on gene expression I used a design model matrix with an interaction between source population pair (ASHN, MYV or SKR), fish type (geothermal or ambient) and rearing temperature (12°C or 18°C), with sex as a covariate. The same design model matrix was used for both *EdgeR* and *LIMMA* analyses. Gene-wise models were then fitted to the count data using the design model matrices.

In *EdgeR* a negative binomial generalized linear model was fitted to each gene. In this process, the variance of gene counts is assumed to depend on the negative binomial dispersion and the quasi-likelihood dispersion (Ren and Kuan, 2020). The negative binomial dispersion represents the variability of the biological system, while the quasi-likelihood dispersion represents gene-specific variability greater or lower than the overall level, capturing both biological and technical sources of variability (Lun et al., 2016). *EdgeR's estimateDisp* function was used to fit a mean-dispersion trend across all genes in the dataset to estimate the negative binomial dispersion (Lun et al., 2016; Ren and Kuan, 2020). An empirical Bayes approach was then used to estimate the quasi-likelihood dispersion and a generalized linear model accommodating the design model matrix was fitted using *glmQLFit* (Lun et al., 2016; Ren and Kuan, 2020).

This method uses the quasi-likelihood (QL) methods developed by Lund et al., (2012) to account for gene-specific variation from biological and technical sources (Lun et al., 2016).

Normalized read counts were prepared for linear modelling in *LIMMA* by performing a voom transformation. The voom method incorporates the mean-variance relationship of the log-counts into the precision weights for each observation (Law et al., 2014, 2018). Next, a linear model was fitted to each gene using *lmFit* (Law et al., 2018).

4.2.3.5 Design of contrasts to test experimental questions.

The questions of this experiment were then addressed by applying tests to the fitted models using contrasts designed to answer each question. Contrasts describe the linear combinations of parameters that should be used to calculate the differences between groups of interest (Law et al., 2020). For example, a contrast of A – B would calculate the difference in means between A and B, thus allowing for an assessment of differential expression between A and B.

To address question 1, and test for consistent divergence between geothermal and ambient sticklebacks, I used a two-step process. First, I tested for differential expression (DE) between ambient and geothermal fish *within* each population pair. I used contrasts where the geothermal fish of each population pair were contrasted against the ambient fish from the same population pair (e.g., "ASHN ambient – ASHN geothermal"). In order to allow for the identification of robust divergence, where differential expression between geothermal and ambient fish was not temperature dependent, I created contrasts for each rearing temperature (i.e., "ASHN ambient at 12°C - ASHN geothermal at 12°C" and "ASHN ambient at 18°C – ASHN geothermal at 18°C"). This resulted in six contrasts (see Table 4-3 for pairwise contrasts). Next, I tested for DE between geothermal and ambient fish *across* population pairs by contrasting the geothermal fish of each population pair and the ambient fish of the remaining population pairs (e.g., "ASHN geothermal - SKR ambient" and "ASHN geothermal – MYV ambient"). Once again, I created these contrasts for each rearing temperature, creating twelve contrasts (see Table 4-3).

Question 2, testing for adaptive divergence in geothermal and ambient stickleback plasticity, was investigated using contrasts where gene expression at

18°C was contrasted against gene expression at 12°C for each morph within each population pair (e.g., ASHN ambient at 12°C – ASHN ambient at 18°C), resulting in six contrasts (see Table 4-3 for pairwise contrasts). These contrasts were then used to test for DE in these comparisons.

4.2.3.6 Hypothesis testing

In *EdgeR*, DE was tested for each contrast using quasi-likelihood F-tests (using the function *glmQLFTest)* (Lun et al., 2016). In *LIMMA*, DE was tested for each gene in the model by using *eBayes* to perform a moderated F-statistic test and rank genes by evidence of DE for each contrast (Law et al., 2018). The top-ranked genes for each contrast were then extracted using *topTable*. For all of these analyses the false discovery rate (FDR) was controlled using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). This was done in *EdgeR* and *LIMMA* using *TopTags* and *topTable* respectively. Genes were considered to be differentially expressed (DE) when the resulting adjusted p value was below 0.05.

The reliability of both *EdgeR* and *LIMMA* analysis methods was assessed by testing for a correlation between the results of each method. I assessed whether *EdgeR* and *LIMMA* methods assigned similar levels of significance to the DE of each gene in each analysis by testing the correlation between the *EdgeR* and *LIMMA* adjusted p values for each gene. This correlation test was performed with a Pearson's correlation using *cor* from the *Stats* R packages (R Core Team, 2022). After ensuring that the *EdgeR* and *LIMMA* results correlated with each other, I selected only genes that were found to have significant DE in both *EdgeR* and *LIMMA* analyses for further analysis. Each question was then addressed by assessing the intersection of divergent genes in each pairwise contrast.

Robust divergence between geothermal and ambient fish was assessed for each population pair by intersecting genes that were DE at 12°C and 18°C. Genes that were DE at both 12°C and 18°C, and with DE in the same direction (e.g., upregulated in geothermal fish) at both rearing temperatures, were considered to be robustly divergent. Genes found to be robustly divergent in multiple population pairs were considered to be evidence of consistent, and therefore likely adaptive, divergence. Divergence between geothermal and ambient fish between population pairs was further assessed by intersecting genes that were differentially expressed in the same direction at both rearing

temperatures. Consistently divergent genes were then assessed by intersecting these gene lists to find genes that were consistently differentially expressed between each population pair's geothermal fish and the ambient fish of all population pairs.

Adaptive divergence in geothermal and ambient plastic responses was assessed by first finding genes that were plastic in either the geothermal or the ambient fish of each population pair, but not in both. Genes that are uniquely plastic in geothermal fish or uniquely plastic in ambient fish suggest divergence in plastic responses between geothermal and ambient fish. Evidence for adaptive divergence in plastic responses was then tested for by finding genes that are divergent in plasticity across multiple population pairs. This was done by intersecting these lists of uniquely plastic genes between population pairs to find genes that were consistently uniquely plastic in the same direction in geothermal fish. This was repeated to test for consistent divergent plastic in ambient fish.

4.2.3.7 Gene ontology analysis

Relevant lists of DE genes for each analysis (geothermal-ambient divergence within and between population pairs, geothermal and ambient plastic responses within and across population pairs) were prepared for ontology analysis by converting Ensembl Stickleback gene codes to Ensembl Zebrafish gene codes using *biomaRt* (Durinck et al., 2009; Kinsella et al., 2011). These zebrafish gene codes were then used for gene ontology and enrichment analyses using Metascape (Zhou et al., 2019) (minimum overlap 3, p value cut-off 0.01 and minimum enrichment 1.5). I used background gene sets of only the expressed genes (non-zero total read) for each tissue. As gene ontology analysis relies on an accurate assessment of whether a particular gene ontology is overrepresented in a dataset, the use of a background gene set specific to the data-set can reduce sampling bias (Timmons et al., 2015).

4.3 Results

4.3.1 Gene expression data

Samples were sequenced with an average of ~23M read pairs and an average read length of 99 base pairs per sample. Alignment rates averaged approximately 70%, with an average of 22,135,426 aligned reads per sample. However, 91 samples had less than 10M reads and were omitted from the

analysis. After this selection, 130 brain and 163 liver samples remained (Table 4-2) with the SKR group being disproportionately affected (Table 4-2).

4.3.2 Gene expression divergence between geothermal and ambient sticklebacks

4.3.2.1 Robust geothermal-ambient divergence within population pairs

Divergence between geothermal and ambient fish within population pairs was found to be strongest in the ASHN geothermal population pair, with a greater number of robust DE genes between geothermal and ambient fish in both

brain (

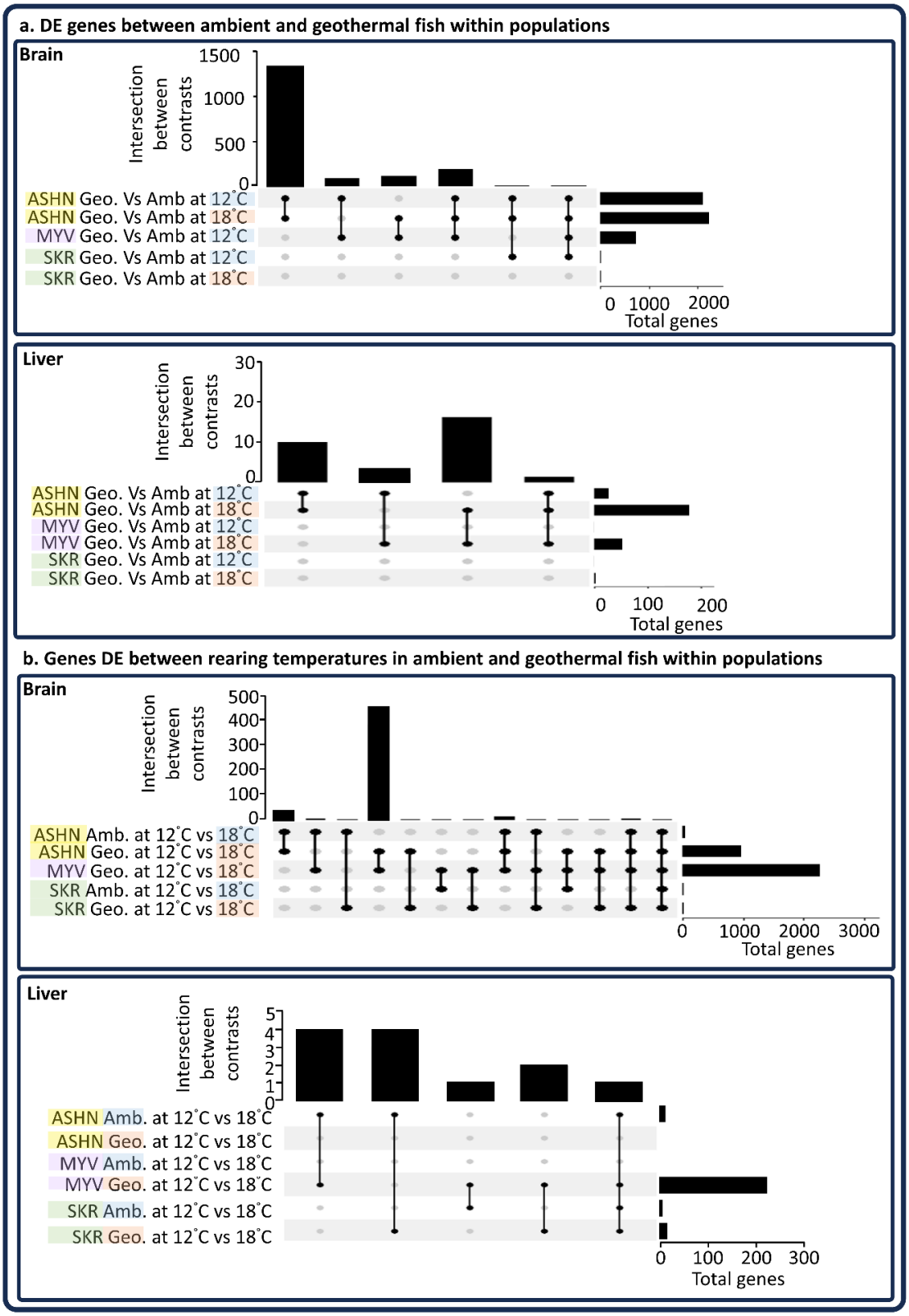

a. DE genes between ambient and geothermal fish within populations
Brain
Intersection between contrasts
1500
1000
500
0
ASHN Geo. Vs Amb at 12˚C
ASHN Geo. Vs Amb at 18˚C
MYV Geo. Vs Amb at 12˚C
SKR Geo. Vs Amb at 12˚C
SKR Geo. Vs Amb at 18˚C
0 1000 2000
Total genes
Liver
Intersection between contrasts
30
20
10
0
ASHN Geo. Vs Amb at 12˚C
ASHN Geo. Vs Amb at 18˚C
MYV Geo. Vs Amb at 12˚C
MYV Geo. Vs Amb at 18˚C
SKR Geo. Vs Amb at 12˚C
SKR Geo. Vs Amb at 18˚C
0 100 200
Total genes
b. Genes DE between rearing temperatures in ambient and geothermal fish within populations
Brain
Intersection between contrasts
500
400
300
200
100
0
ASHN Amb. at 12˚C vs 18˚C
ASHN Geo. at 12˚C vs 18˚C
MYV Geo. at 12˚C vs 18˚C
SKR Amb. at 12˚C vs 18˚C
SKR Geo. at 12˚C vs 18˚C
0 1000 2000 3000
Total genes
Liver
Intersection between contrasts
5
4
3
2
1
0
ASHN Amb. at 12˚C vs 18˚C
ASHN Geo. at 12˚C vs 18˚C
MYV Amb. at 12˚C vs 18˚C
MYV Geo. at 12˚C vs 18˚C
SKR Amb. at 12˚C vs 18˚C
SKR Geo. at 12˚C vs 18˚C
0 100 200 300
Total genes

Figure 4-2a i) and liver (

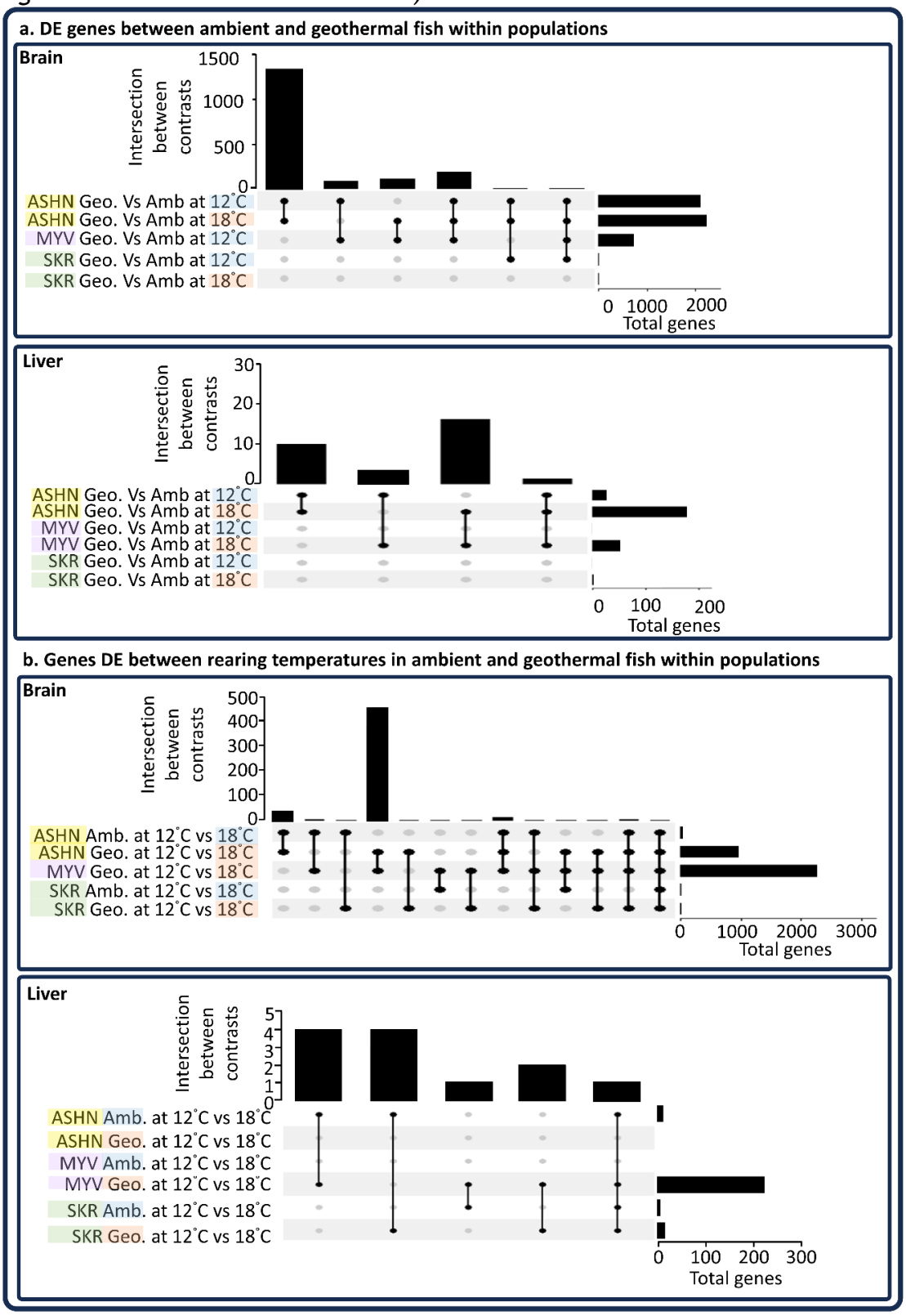


Figure 4-2a v) tissue. The MYV population pair also showed a relatively large number of DE genes at 12°C in the brain (Table 4-3,

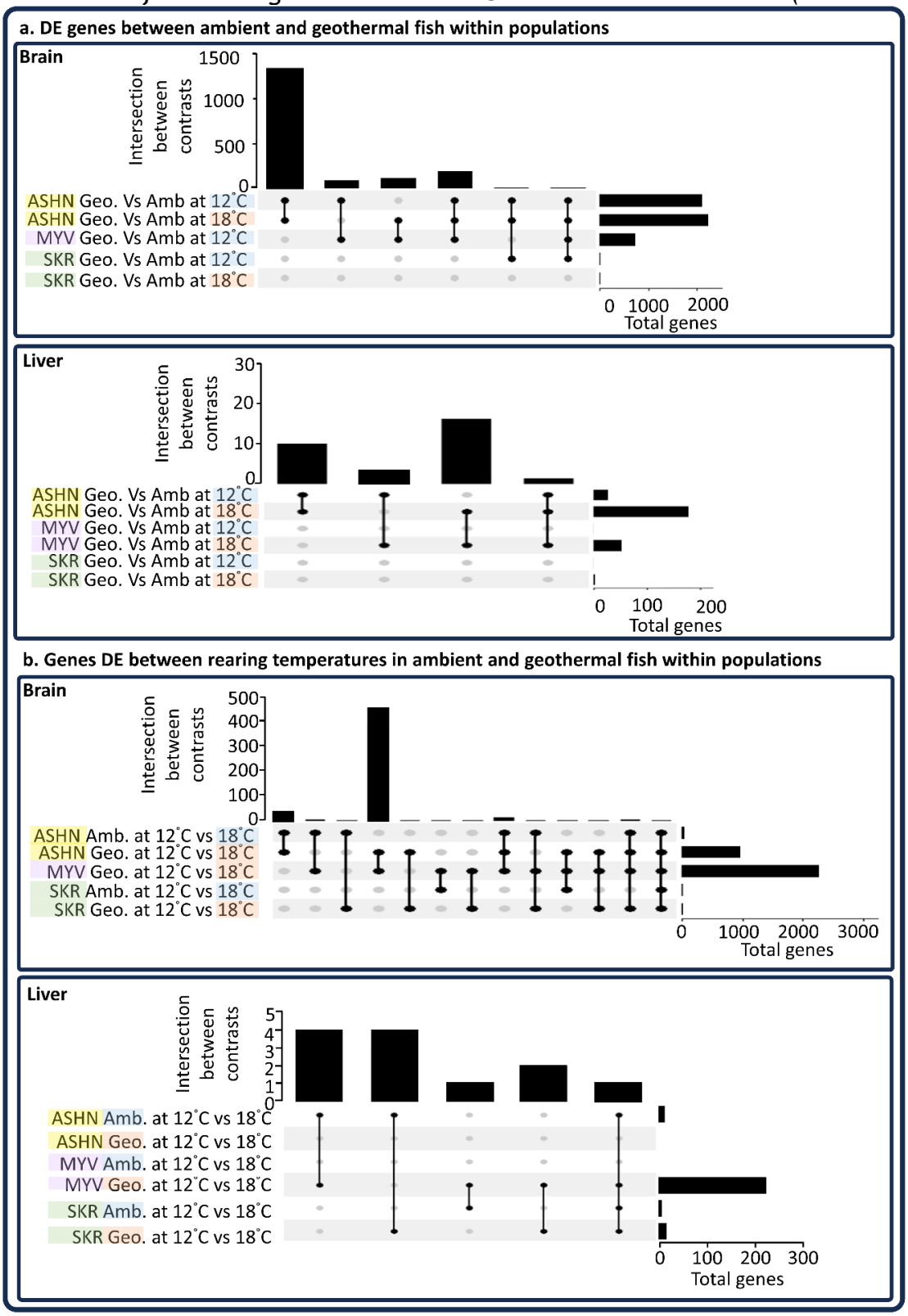


Figure 4-2a iii), but no robust divergence in the liver (

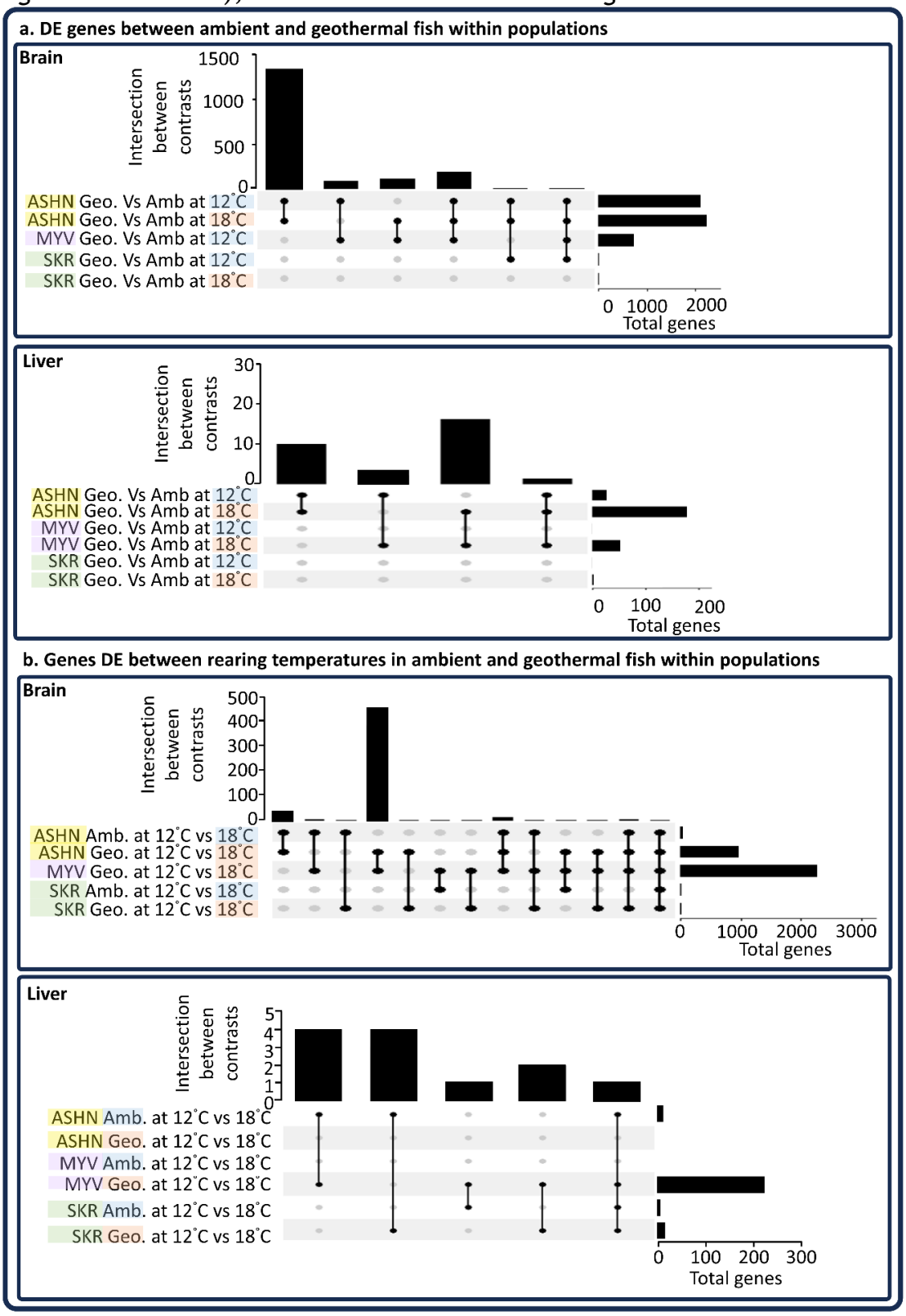


Figure 4-2a vi). Divergence between geothermal and ambient brains in MYV and ASHN population pairs involved similar gene ontologies, with strong enrichment for genes relating to metabolic processes (GO:0008152) (Figure 4-3a).

4.3.2.2 Consistent, robust geothermal-ambient divergence across population pairs

For brain tissue robust divergence in gene expression between geothermal and ambient fish had consistencies across ASHN and MYV population pairs. The SKR population pair showed no robust divergence and therefore little consistency with either ASHN or MYV population pairs. Out of the 1330 robustly divergent genes in ASHN brains, and 630 genes that were divergent at 12^{o}C in MYV brains, I found 231 genes that were divergent in both population pairs. Of these genes, 213 were conserved in the direction of the log fold change between geothermal and ambient brains, with 73 downregulated and 140 upregulated in geothermal fish. I further tested for consistency in geothermal-ambient divergence across the ASHN and MYV population pairs by testing for DE between geothermal and ambient fish from different population pairs. Of the 213 genes that were consistently divergent between geothermal and ambient fish within both MYV and ASHN population pairs, 148 were consistently divergent between ambient and geothermal fish of either population pair. I found 46 genes that were upregulated and 102 that were downregulated in the brains of geothermal fish. Consistently divergent genes that were upregulated in geothermal fish were most strongly enriched for metabolic processes, particularly the glucose metabolic process (GO:0006006) (Table 4-4, Figure 4-3b). Other notable enriched gene ontologies included response to hypoxia (GO:0001666) and regulation of nervous system development (Figure 4-3b). Consistently divergent genes that were downregulated in geothermal fish were enriched for protein folding (GO:0006457).

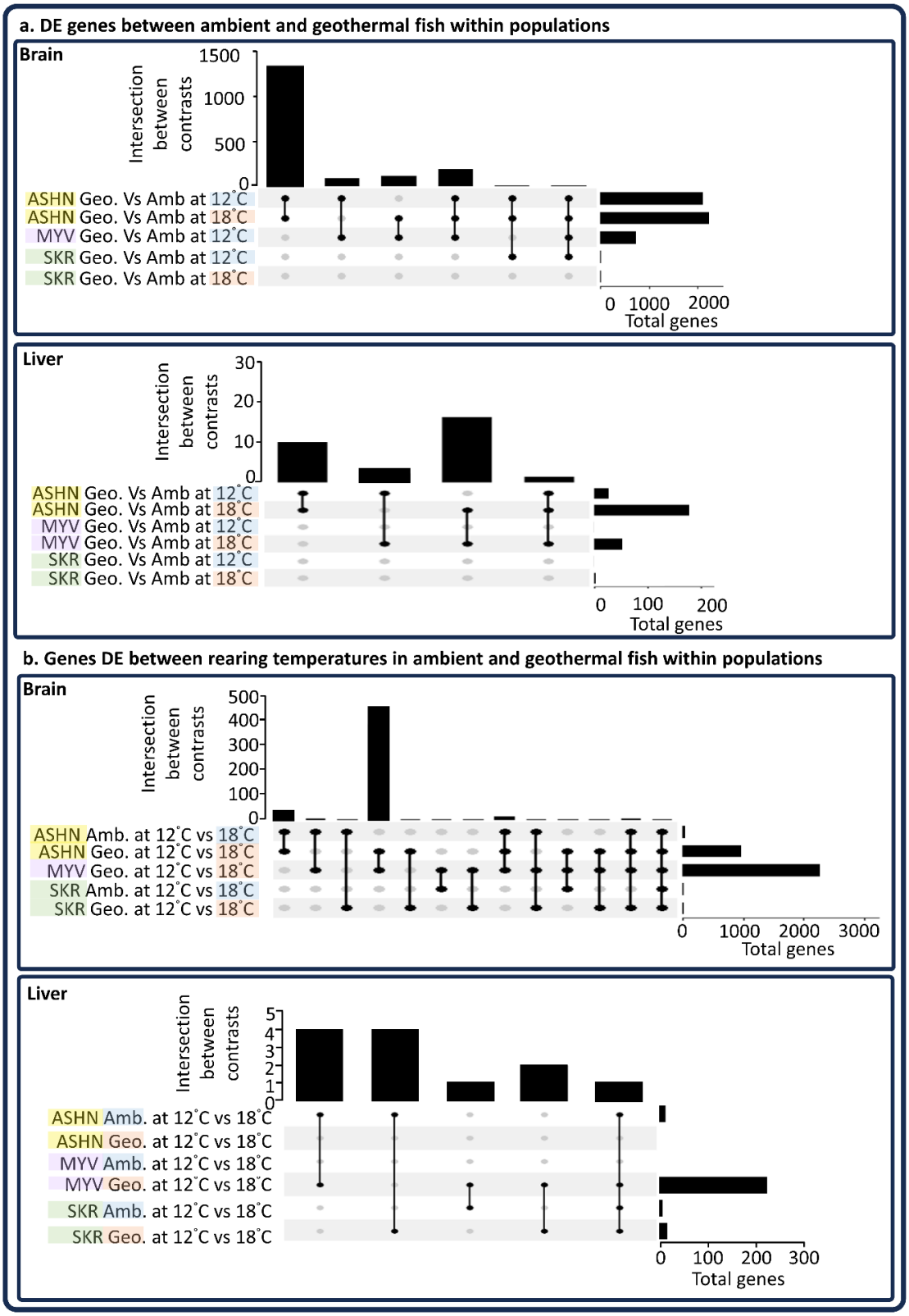


Figure 4-2 Upset plots (R package "ComplexHeatmaps") showing the number of genes (and intersecting genes) differentially expressed in each analysis contrasting gene expression a) between ambient and geothermal sticklebacks within population pairs and b) between rearing temperatures (geothermal and ambient plastic responses) within population pairs

Table 4-3 Numbers of DE genes found for each analysis, including EdgeR and LIMMA results.

		Brain (*EdgeR:13142/LIMMA:13144*)			Liver (*EdgeR:9583/LIMMA:9567*)		
Analysis	**Comparison *(EdgeR/Limma)***	**Total DE genes**	**Direction**		**Total DE genes**	**Direction**	
Within population Geo. vs Amb. fish DE	**Pairwise contrasts**		**Downregulated in Geo. fish**	**Upregulated in Geo. fish**		**Downregulated in Geo. fish**	**Upregulated in Geo. fish**
	MYV Geo. vs Amb. at 12˚C	630 (740/1059)	329 (416/369)	301 (324/690)	0(4/0)	0 (2/0)	0 (2/0)
	MYV Geo. vs Amb. at 18˚C	-	-	-	54 (83/97)	35 (44/53)	19 (39/44)
	SKR Geo. vs Amb. at 12˚C	2 (14/2)	2 (12/2)	0 (2/0)	0 (2/0)	0 (1/0)	0 (1/0)
	SKR Geo. vs Amb. at 18˚C	2 (4/2)	1 (3/1)	1 (1/1)	1 (1/16)	0 (0/3)	1 (1/13)
	ASHN Geo. vs Amb. at 12˚C	2122 (3088/2437)	1083 (1307/1384)	AA49 (1781/1053)	20 (59/58)	12 (18/46)	8 (41/12)
	ASHN Geo. vs Amb. at 18˚C	2255 (3429/2440)	1098 (1350/1279)	1156 (2079/1161)	175 (255/291)	93 (111/185)	82 (144/106)
Between population Geo. vs Amb. fish DE	**Pairwise contrasts**		**Downregulated in Geo. fish**	**Upregulated in Geo. fish**		**Downregulated in Geo. fish**	**Upregulated in Geo. fish**
	MYV Geo. vs ASHN Amb. at 12˚C	1013 (1920/MG49)	453 (770/476)	560 (1150/573)	29 (47/46)	27 (35/39)	2 (12/7)
	MYV Geo. vs ASHN Amb. at 18˚C	2881 (4147/3080)	1390 (1540/1583)	1491 (2607/1497)	252 (394/403)	122 (139/231)	130 (255/172)
	MYV Geo. vs SKR Amb. at 12˚C	181 (278/208)	71 (126/89)	110 (152/119)	19 (26/32)	17 (24/29)	2 (2/3)
	MYV Geo. vs SKR Amb. at 18˚C	113 (399/120)	65(94/71)	48 (305/49)	166 (218/423)	57 (72/93)	109 (146/330)
	SKR Geo. vs ASHN Amb. at 12˚C	0 (5/0)	0 (3/0)	0 (2/0)	0 (5/0)	0 (2/0)	0 (3/0)
	SKR Geo. vs ASHN Amb. at 18˚C	202 (704/202)	65 (187/65)	137 (517/137)	0 (1/1)	0 (0/1)	0 (1/0)
	SKR Geo. vs MYV Amb. at 12˚C	435 (771/548)	205 (474/205)	230 (297/343)	95 (176/124)	30 (68/44)	65 (108/80)
	SKR Geo. vs MYV Amb. at 18˚C	-	-	-	186 (252/272)	43 (88/61)	143 (164/211)
	ASHN Geo. vs MYV Amb. at 12˚C	1875 (2MA3/2783)	925 (1016/1205)	950 (1026/1578)	187 (362/277)	100 (125/181)	87 (237/96)
	ASHN Geo. vs MYV Amb. at 18˚C	-	-	-	111 (140/168)	56 (67/93)	55 (73/75)
	ASHN Geo. vs SKR Amb. at 12˚C	1113 (1920/MG49)	574 (610/896)	539 (605/599)	3 (3/32)	2 (2/27)	1 (1/5)
	ASHN Geo. vs SKR Amb. at 18˚C	17 (22/23)	15 (16/21)	2 (6/2)	173 (261/319)	64 (112/95)	109 (149/224)
Geo. vs Amb. plasticity	**Pairwise contrasts**		**Downregulated at 18˚C**	**Upregulated at 18˚C**		**Downregulated at 18˚C**	**Upregulated at 18˚C**
	MYV Geo. at 12˚C vs 18˚C	2269 (2585/2812)	1002 (1019/1440)	1267 (1566/1372)	221 (342/268)	76 (92/172)	145 (250/196)
	MYV Amb. at 12˚C vs 18˚C	-	-	-	0 (1/0)	0 (1/0)	0 (0/0)
	SKR Geo. at 12˚C vs 18˚C	24 (61/25)	20 (45/21)	4 (16/4)	13 (29/19)	8 (19/11)	5 (10/8)
	SKR Amb. at 12˚C vs 18˚C	6 (22/7)	4 (12/4)	2 (10/3)	4 (7/8)	2 (3/6)	2 (4/2)
	ASHN Geo. at 12˚C vs 18˚C	980 (1SA5/1802)	460 (484/778)	520 (598/1024)	0 (11/0)	0 (5/0)	0 (6/0)
	ASHN Amb. at 12˚C vs 18˚C	45 (327/45)	23 (188/23)	22 (139/22)	10 (23/12)	4 (16/5)	6 (7/7)

Table 4-4 Enriched gene ontology terms in genes DE between geothermal and ambient fish brains in both ASHN and MYV populations

Parent GO	*GO term*	*Reg*	*Log(q)*	*Log(P)*	*Z*	
GO:0SA2152 metabolic process	glucose metabolic process (GO:0006006)	↑	-0.54	-4.38	8.56	4
	carboxylic acid metabolic process (GO:0019752)	↑	-0.54	-4.19	5.20	11
	oxoacid metabolic process (GO:0043436)	↑	-0.54	-4.17	5.17	11
	organic acid metabolic process (GO:0006082)	↑	-0.54	-4.08	5.08	11
	pyruvate metabolic process (GO:0006090)	↑	-0.19	-3.55	6.52	4
	hexose metabolic process (GO:0019318)	↑	-0.19	-3.43	6.26	4
	monocarboxylic acid metabolic process (GO:0032787)	↑	-0.19	-3.38	4.87	7
	monosaccharide metabolic process (GO:0005996)	↑	-0.10	-3.19	5.74	4
	sulfur compound biosynthetic process (GO:0044272)	↑	-0.10	-3.09	5.55	4
	protein folding (GO:0006457)	↓	-0.10	-3.05	5.45	4
	glycolytic process (GO:0006096)	↑	0.00	-2.78	5.70	3
	generation of precursor metabolites and energy (GO:0006091)	↑	0.00	-2.75	4.04	7
	sulfur compound metabolic process (GO:0006790)	↑	0.00	-2.66	4.30	5
	protein maturation (GO:0051604)	↓	0.00	-2.22	3.32	7
	carbohydrate catabolic process (GO:0016052)	↑	0.00	-2.18	4.25	3
	RNA splicing (GO:0008380)	↓	0.00	-2.18	3.78	4
	aerobic respiration (GO:0009060)		0.00	-2.10	3.65	4
	regulation of RNA stability (GO:0043487)	↑	0.00	-2.05	3.95	3
GO:0032501 multicellular organismal process	mechanoreceptor differentiation (GO:0042490)	↑	0.00	-2.49	4.97	3
	regulation of nervous system development (GO:0051960)	↑	0.00	-2.12	3.67	4
GO:0050896 response to stimulus	response to hypoxia (GO:0001666)	↑	0.00	-2.66	5.40	3
	response to decreased oxygen levels (GO:0036293)	↑	0.00	-2.63	5.30	3
	response to oxygen levels (GO:0070482)	↑	0.00	-2.56	5.13	3
none	proton-transporting two-sector ATPase complex (GO:0016469)	↓	-0.19	-3.42	7.57	3
none	spliceosomal complex (GO:0005681)	↓	-0.10	-3.04	5.42	4
none	proton transmembrane transporter activity (GO:0015078)	↓	-0.07	-2.96	6.20	3
none	proton-transporting two-sector ATPase complex, proton-transporting domain (GO:0033177)		0.00	-2.87	5.91	3
none	mRNA 3'-UTR binding (GO:0003730)		0.00	-2.66	4.67	4
none	single-stranded RNA binding (GO:0003727)		0.00	-2.63	5.28	3
none	ATPase complex (GO:1904949)	↓	0.00	-2.39	4.73	3
none	inner mitochondrial membrane protein complex (GO:0098800)	↓	0.00	-2.32	4.55	3
none	SCF ubiquitin ligase complex (GO:0019005)		0.00	-2.10	4.06	3

4.3.3 Gene expression plasticity divergence

4.3.3.1 Divergent plasticity within population pairs

Plasticity in response to temperature was present, with the brains of geothermal fish being more plastic than ambient fish in the ASHN and SKR population pairs (Figures 2c i and ii). While I could not assess the plastic response of ambient brains in the MYV group, the plastic response of MYV geothermal fish brains involved more than twice the number of genes of any

other group (Table 4-3,

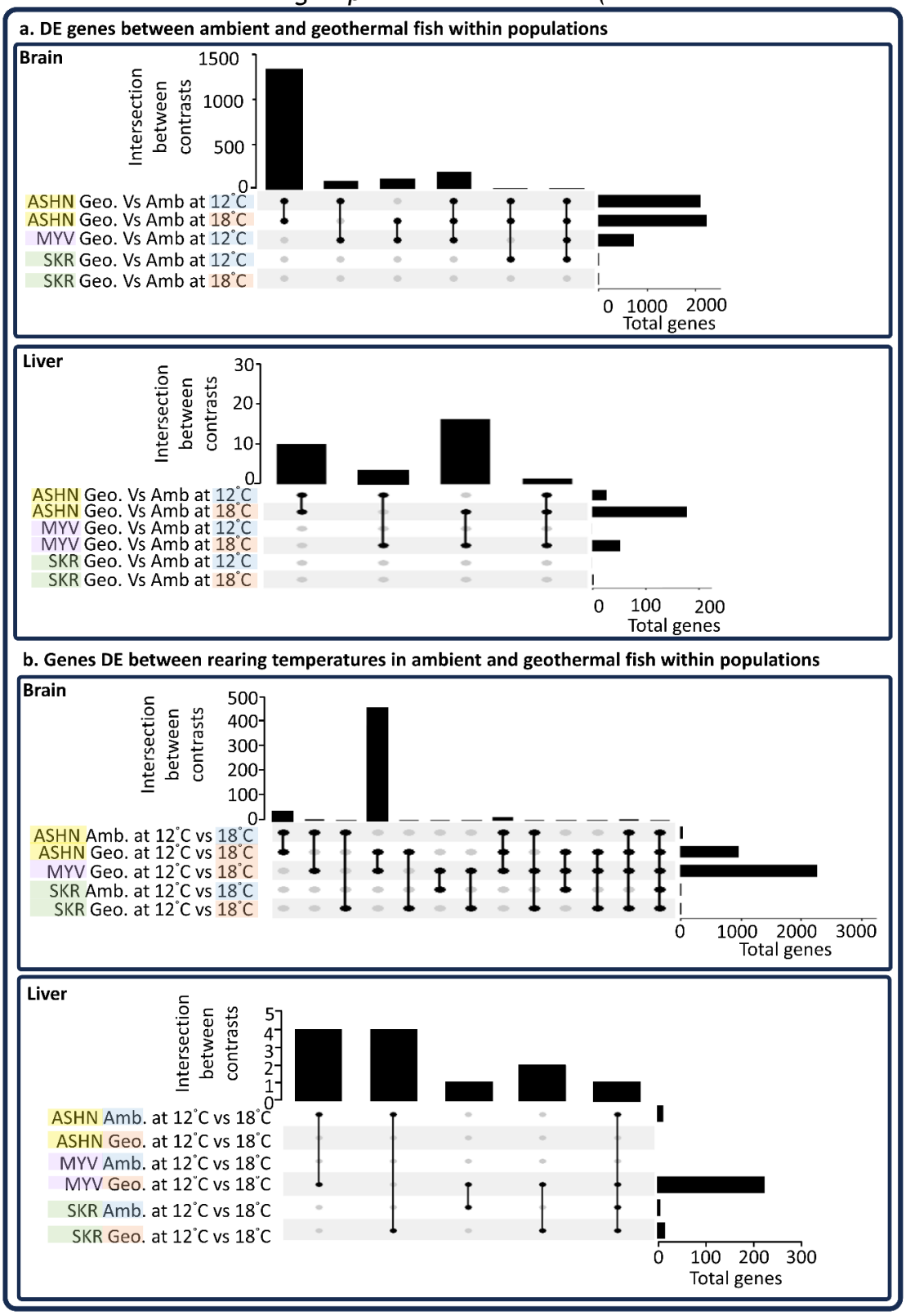


Figure 4-2c iii). The ASHN and MYV geothermal plastic response in the brain both involve genes relating to metabolic process (GO:0008152) and immune system process (GO:0002376) gene ontologies (Figure 3c).

I also found greater number of plastic genes in geothermal fish in the livers of SKR and MYV fish, however, the livers of ASHN ambient fish were more

plastic than ASHN geothermal fish, with no divergent plastic response in geothermal fish at all (Figure 2c). Divergent geothermal plasticity in the livers of SKR and MYV fish showed no similarity in enriched gene ontologies (Figure 4-3c)

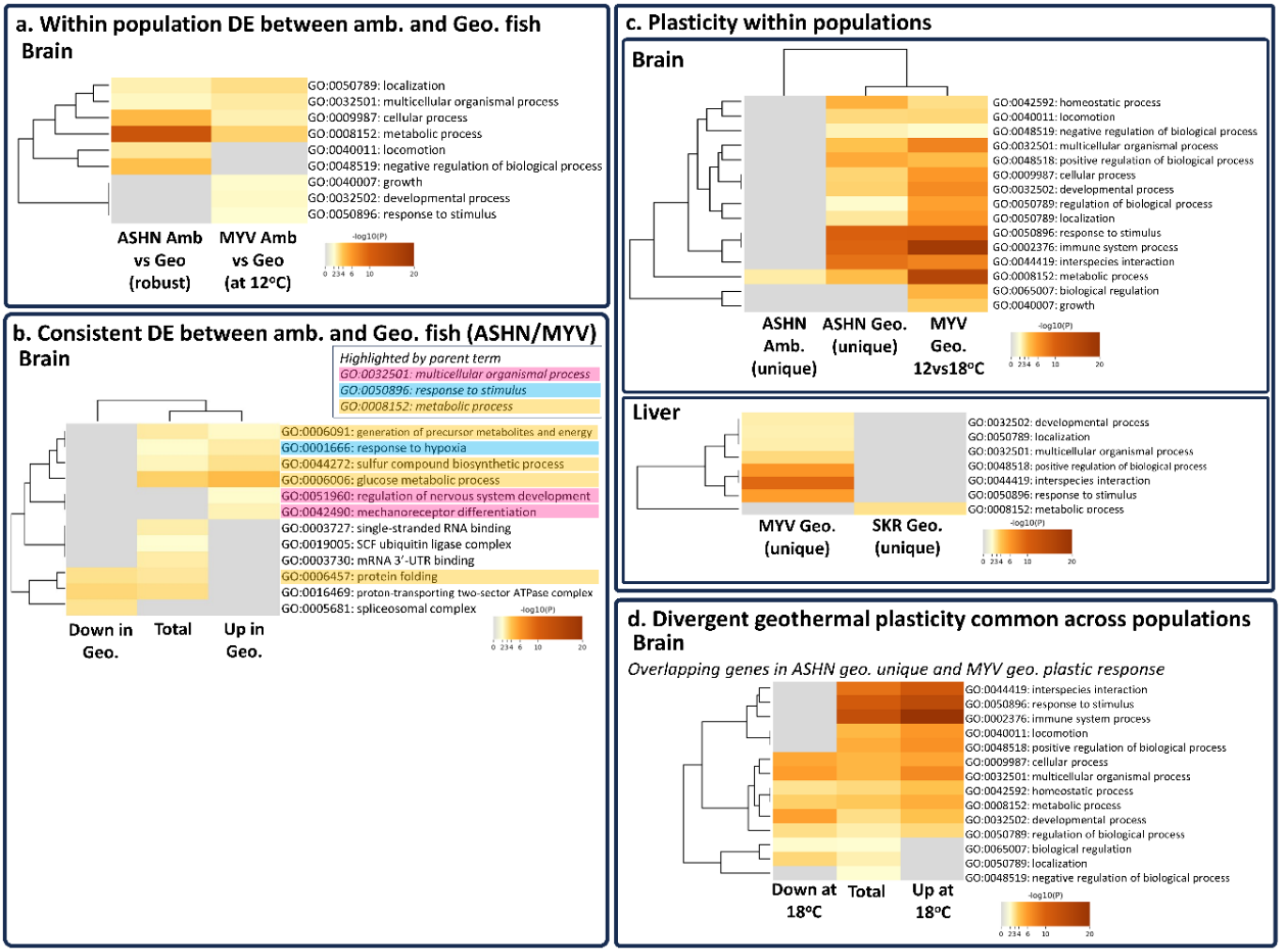


Figure 4-3 Heatmaps showing gene ontology terms enriched in differentially expressed genes in a) comparisons of ambient and geothermal fish within populations, b) genes differentially expressed between ambient and geothermal fish in both ASHN and MYV population pairs, c) within population pair plasticity and d) divergent geothermal plasticity in both ASHN and MYV population pairs.

4.3.3.2 Consistent divergent plasticity across population pairs

I found little evidence of a consistent divergence in gene expression plasticity in geothermal and ambient fish across all the three population pairs, with only three genes found to be plastic in geothermal fish (and not ambient fish) for the brains across the three population pairs, without also being plastic in ambient fish (

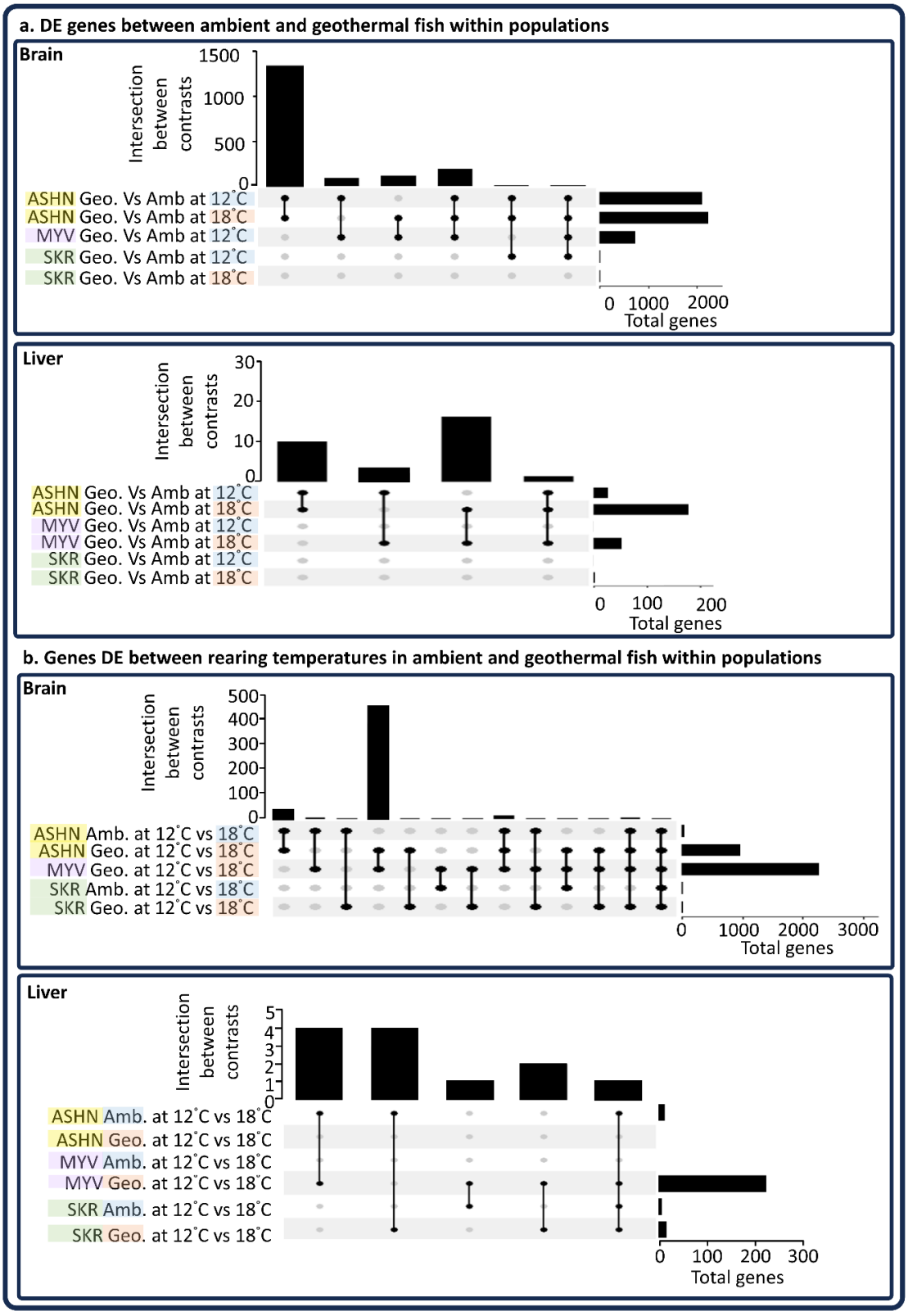


Figure 4-2d ii). These three genes were srsf6b (predicted to enable mRNA binding activity), dazap2 (expressed in central nervous system with various functions) and fkbp3 (enables peptidyl-prolyl cis/trans isomerase activity, involved in protein folding).

However, I also tested for consistency in divergent plastic responses in geothermal and ambient fish between the MYV and ASHN population pairs. In the brain I found 448 genes that were plastic in the same direction in both MYV geothermal fish and ASHN geothermal fish, but not in ASHN ambient fish. 265 of these genes were upregulated and 183 downregulated at 18^{o}C. This MYV-ASHN consistent plastic response to the warmer rearing temperature is most strongly enriched for an upregulation in immune system processes (GO:0002376) (Figure 4-3d, Table 4-5). These processes included a number of aspects of the innate immune system, including lymphocytes (activation and proliferation), neutrophils (migration and chemotaxis), macrophages (differentiation, activation, chemotaxis and migration) and the toll-like receptor signalling pathway (Table 4-5). Components of the active immune system were also upregulated, including T cells (activation) and antigens (processing, presentation and signalling pathway) (Table 4-5). I found few genes that were plastic in

ambient fish, but not in geothermal fish in most comparisons (

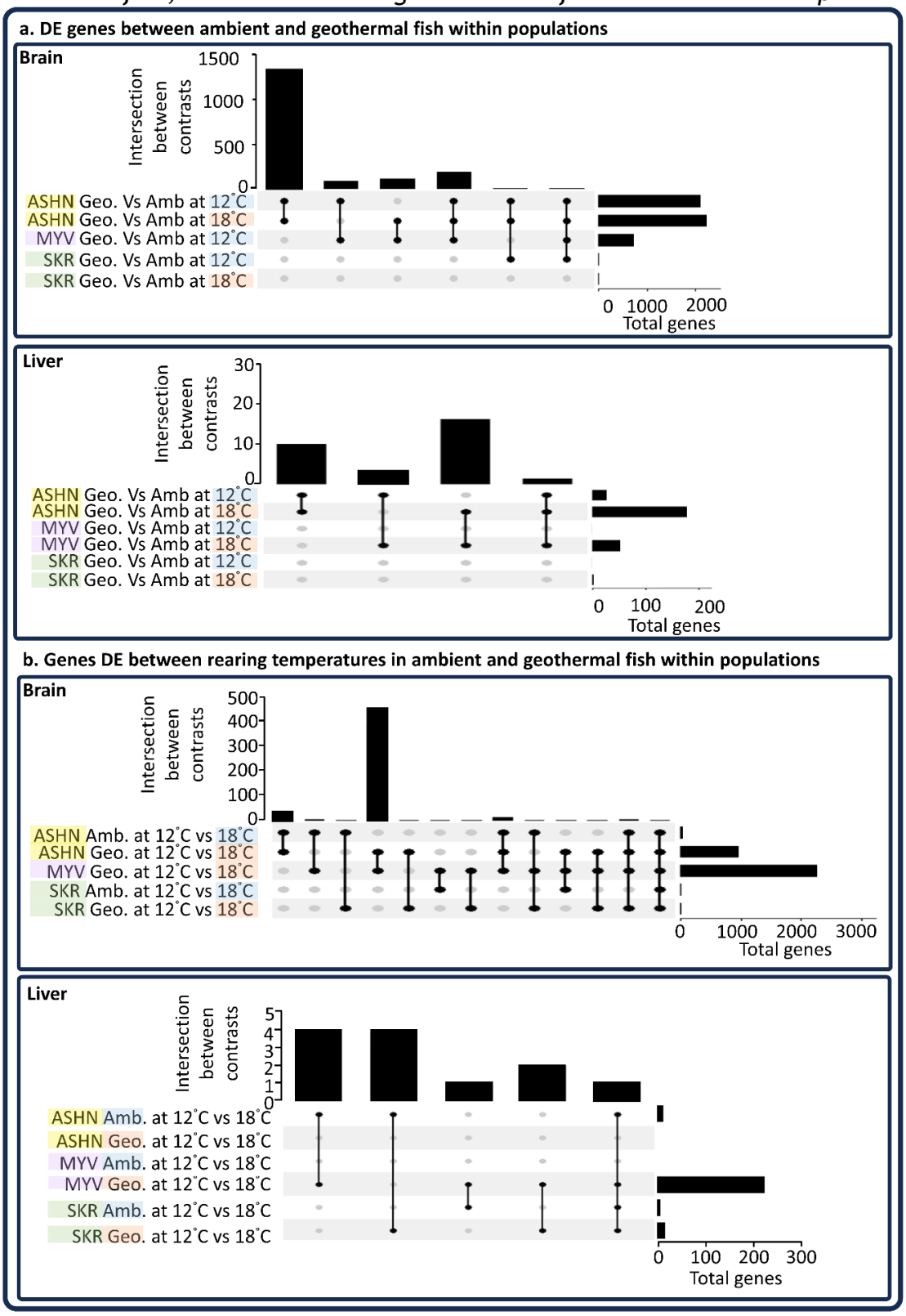


Figure 4-2c, Table 4-2). The few genes that were uniquely plastic in ambient fish did not overlap between population pairs, therefore suggesting little consistency

in the divergence in the ambient fish plastic response (

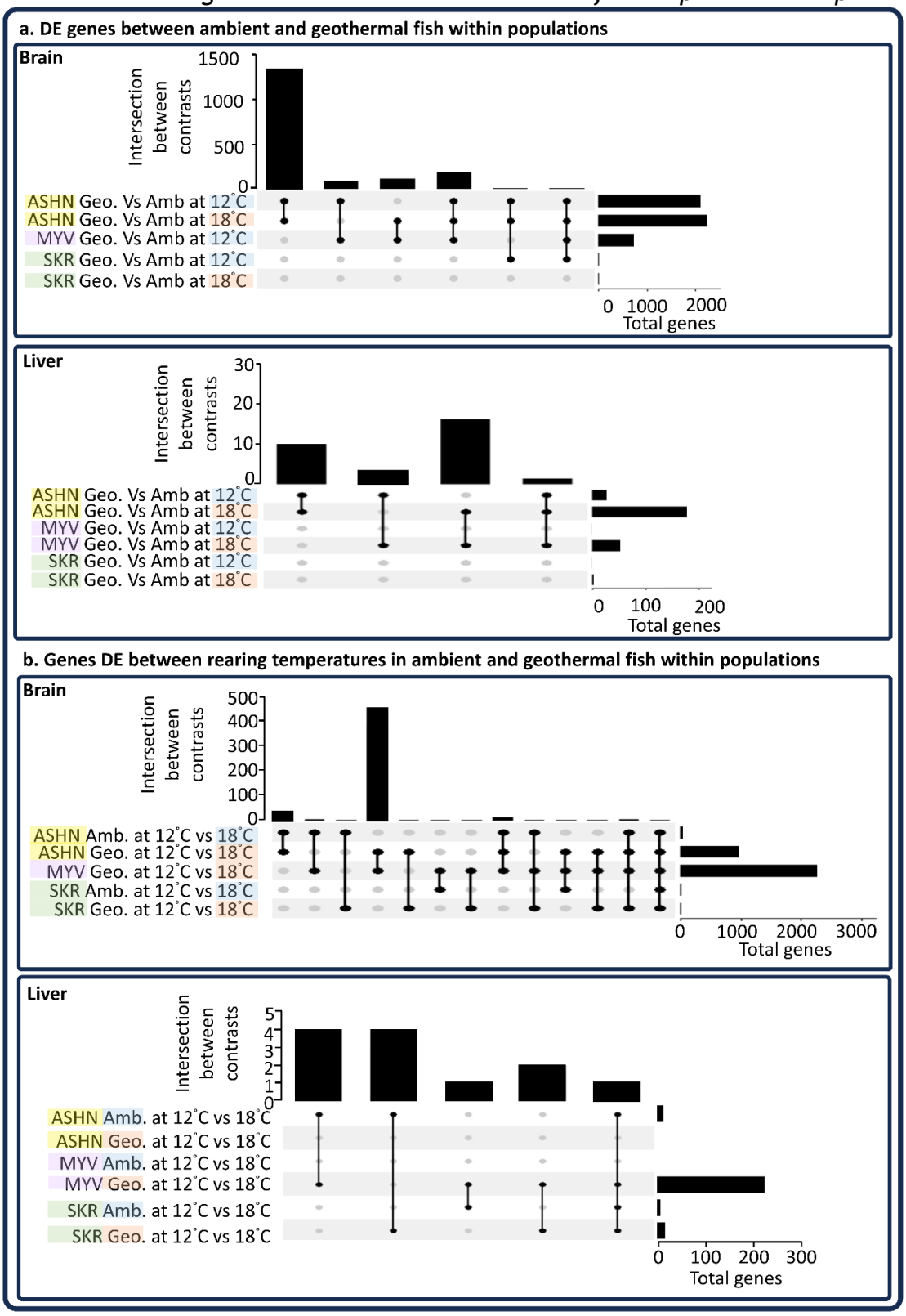


Figure 4-2d).

There was little crossover in plastic genes between SKR and MYV geothermal, with only a single gene, *fat3a* (related to calcium ion binding activity and cartilage morphogenesis) found in both responses. There was also

little similarly between the gene ontology of the MYV and SKR geothermal plastic responses in the liver (Figure 4-3c).

Table 4-5 Enriched gene ontology terms in genes consistently plastic in both ASHN and MYV geothermal brains.

Parent GO	*GO term*	*Reg.*	*Log(q)*	*Log(P)*	*Z*	
GO:0002376 immune system process	immune system process (GO:0002376)	↑	-16.43	-20.57	13.76	39
	reg. of immune system process (GO:0002682)	↑	-10.70	-14.37	12.03	24
	immune response (GO:0006955)	↑	-8.26	-11.70	10.91	19
	+ve reg. of immune system process (GO:0002684)	↑	-7.28	-10.31	10.65	15
	reg. of immune response (GO:0050776)	↑	-7.28	-10.31	10.65	15
	+ve reg. of immune response (GO:0050778)	↑	-7.78	-11.02	12.16	13
	leukocyte activation (GO:0045321)	↑	-5.61	-8.45	10.01	11
	activation of immune response (GO:0002253)	↑	-6.36	-9.28	11.57	10
	immune response-activating signaling pathway (GO:0002757)	↑	-5.13	-7.77	10.73	8
	immune response-regulating signaling pathway (GO:0002764)	↑	-5.13	-7.77	10.73	8
	myeloid leukocyte migration (GO:0097529)	↑	-2.63	-4.81	7.34	6
	leukocyte migration (GO:0050900)	↑	-2.28	-4.36	6.61	6
	reg. of hemopoiesis (GO:1903706)	↑	-2.09	-4.13	6.25	6
	lymphocyte activation (GO:0046649)	↑	-1.83	-3.79	5.73	6
	neutrophil migration (GO:1990266)	↑	-2.18	-4.24	6.98	5
	granulocyte migration (GO:0097530)	↑	-2.09	-4.13	6.79	5
	immune response-regulating cell surface receptor signaling (GO:0002768)	↑	-1.44	-3.87	6.17	5
	immune effector process (GO:0002252)	↑	-1.66	-3.60	5.85	5
	reg. of leukocyte activation (GO:0002694)	↑	-1.28	-3.08	4.97	5
	myeloid leukocyte activation (GO:0002274)	↑	-2.98	-5.22	10.20	4
	+ve reg. of immune effector process (GO:0002699)	↑	-2.37	-4.46	8.40	4
	reg. of immune effector process (GO:0002697)	↑	-1.94	-3.95	7.24	4
	reg. of myeloid cell differentiation (GO:0045637)	↑	-1.55	-3.45	6.20	4
	antigen receptor-mediated signaling pathway (GO:0050851)	↑	-1.02	-3.34	5.82	4
	immune response-activating cell surface receptor signaling (GO:0002429)	↑	-0.69	-2.90	5.00	4
	reg. of lymphocyte activation (GO:0051249)	↑	-0.94	-2.60	4.53	4
	-ve reg. of immune system process (GO:0002683)	↑	-0.75	-2.33	4.04	4
	macrophage activation (GO:0042116)	↑	-2.37	-4.45	10.21	3
	reg. of lymphocyte proliferation (GO:0050670)	↑	-1.46	-3.34	7.02	3
	leukocyte activation involved in immune response (GO:0002366)	↑	-1.34	-3.17	6.57	3
	antigen processing and presentation (GO:0019882)	↑	-1.34	-3.17	6.57	3
	cell activation involved in immune response (GO:0002263)	↑	-1.23	-3.02	6.18	3
	macrophage migration (GO:1905517)	↑	-1.13	-2.89	5.85	3
	B cell receptor signaling pathway (GO:0050853)		-0.53	-2.64	5.13	3
	-ve reg. of leukocyte activation (GO:0002695)		-0.13	-2.09	3.97	3
GO:0008152 metabolic process	amide metabolic process (GO:0043603)	↑	-0.76	-2.97	3.49	28
	peptide metabolic process (GO:0006518)	↑	-2.28	-4.36	4.89	18
	translation (GO:0006412)	↑	-2.50	-4.63	5.18	17
	peptide biosynthetic process (GO:0043043)	↑	-2.40	-4.50	5.06	17
	amide biosynthetic process (GO:0043604)	↑	-1.54	-3.44	4.11	17
	cytoplasmic translation (GO:0002181)	↑	-2.47	-4.58	6.53	7
	superoxide metabolic process (GO:0006801)	↑	-2.80	-5.02	8.48	5
	reactive oxygen species metabolic process (GO:0072593)	↑	-1.81	-3.76	6.13	5
	pyruvate metabolic process (GO:0006090)	↓	-1.48	-3.37	5.48	5
	superoxide anion generation (GO:0042554)	↑	-2.52	-4.67	8.90	4
GO:0009987 cellular process	supramolecular fibre organization (GO:0097435)	↑	-1.98	-4.59	4.90	23
	actin filament-based process (GO:0030036)	↑	-1.65	-4.18	4.58	23
	actin cytoskeleton organization (GO:0030036)	↑	-1.44	-3.91	4.39	22
	cell migration (GO:0016477)	↑	-1.87	-3.84	4.48	17
	cell motility (GO:0048870)	↑	-1.61	-3.53	4.20	17
	actin filament organization (GO:0007015)	↑	-3.13	-5.43	6.25	13
	cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	↓	-3.10	-5.38	7.31	8
	organelle fusion (GO:0048284)		-0.08	-2.02	3.05	7
	mitochondrial fusion (GO:0008053)		-0.33	-2.33	4.47	3
GO:0032501 multicellular organismal process	neuron development (GO:0048666)	↓	-3.05	-5.32	5.57	20
	neuron projection development (GO:0031175)	↓	-3.43	-5.86	6.14	18
	cell morphogenesis involved in neuron differentiation (GO:0048667)	↓	-3.03	-5.29	5.90	15
	axon development (GO:0061564)	↓	-3.00	-5.25	5.87	15
	neuron projection morphogenesis (GO:0048812)	↓	-2.75	-4.96	5.60	15
	axonogenesis (GO:0007409)	↓	-2.76	-4.98	5.71	14
	cell activation (GO:0001775)	↑	-4.44	-7.02	8.32	11
	reg. of cell activation (GO:0050865)	↑	-1.23	-3.02	4.88	5
	blood coagulation (GO:0007596)		-0.11	-2.06	3.54	4
	coagulation (GO:0050817)		-0.11	-2.06	3.54	4
	reg. of gliogenesis (GO:0014013)	↑	-1.06	-2.77	5.56	3
GO:0032502 developmental process	cellular component morphogenesis (GO:0032989)	↓	-3.30	-5.68	6.00	18
	cell part morphogenesis (GO:0032990)	↓	-3.19	-5.50	6.00	16
	plasma membrane bounded cell projection morphogenesis (GO:0120039)	↓	-2.75	-4.96	5.60	15

Parent GO	*GO term*	*Reg.*	*Log(q)*	*Log(P)*	*Z*	
	cell projection morphogenesis (GO:0048858)	↓	-2.74	-4.94	5.58	15
	cell morphogenesis (GO:0000902)	↓	-2.03	-4.05	4.76	15
	myeloid cell differentiation (GO:0030099)	↑	-1.57	-3.48	4.57	10
	macrophage differentiation (GO:0030225)	↑	-2.07	-4.10	7.58	4
GO:0SA6MG5 locomotion	cell chemotaxis (GO:0060326)	↑	-3.66	-6.14	8.88	7
	chemotaxis (GO:0006935)	↑	-1.93	-3.93	5.61	7
	taxis (GO:0042330)	↑	-1.93	-3.93	5.61	7
	locomotion (GO:0040011)	↑	-1.65	-3.59	5.15	7
	leukocyte chemotaxis (GO:0030595)	↑	-3.19	-5.50	8.51	6
	neutrophil chemotaxis (GO:0030593)	↑	-1.55	-3.45	6.20	4
	granulocyte chemotaxis (GO:0071621)	↑	-1.47	-3.35	5.99	4
	macrophage chemotaxis (GO:0048246)	↑	-1.46	-3.34	7.02	3
	reg. of blood vessel endothelial cell migration (GO:0043535)	↑	-1.23	-3.02	6.18	3
GO:0042592 homeostatic process	reg. of cellular pH (GO:0030641)	↓	-0.79	-3.02	4.81	5
	reg. of pH (GO:0006885)	↓	-0.66	-2.84	4.52	5
	reg. of erythrocyte differentiation (GO:0045646)	↑	-2.04	-4.06	9.07	3
GO:0044419 biological process involved in interspecies interaction between organisms	interspecies interaction (GO:0044419)	↑	-8.08	-11.44	10.48	20
	response to other organism (GO:0051707)	↑	-7.64	-10.78	10.12	19
	defense response to other organism (GO:0098542)	↑	-7.06	-10.05	10.12	16
	innate immune response (GO:0045087)	↑	-5.13	-7.80	9.23	11
	response to bacterium (GO:0009617)	↑	-3.31	-5.70	7.35	9
	reg. of innate immune response (GO:0045088)	↑	-3.43	-5.87	7.95	8
	defense response to bacterium (GO:0042742)	↑	-1.41	-3.84	5.42	7
	+ve reg. of innate immune response (GO:0045089)	↑	-3.28	-5.64	8.75	6
	activation of innate immune response (GO:0002218)	↑	-3.41	-5.81	10.08	5
	pattern recognition receptor signaling pathway (GO:0002221)	↑	-3.41	-5.81	10.08	5
	innate immune response-activating signaling pathway (GO:0002758)	↑	-3.41	-5.81	10.08	5
	response to virus (GO:0009615)	↑	-1.87	-3.85	6.28	5
	toll-like receptor signaling pathway (GO:0002224)	↑	-2.73	-4.92	9.49	4
	defense response to virus (GO:0051607)	↑	-1.41	-3.26	5.79	4
	defense response to symbiont (GO:0140546)	↑	-1.41	-3.26	5.79	4
GO:0048518 +ve reg. of biological process	+ve reg. of response to stimulus (GO:0048584)	↑	-3.49	-5.94	6.09	20
	+ve reg. of defense response (GO:0031349)	↑	-4.05	-6.55	9.53	7
	+ve reg. of response to external stimulus (GO:0032103)	↑	-2.97	-5.20	7.43	7
	+ve reg. of response to biotic stimulus (GO:0002833)	↑	-3.28	-5.64	8.75	6
GO:0048519 -ve reg. of biological process	-ve reg. of cell activation (GO:0050866)		-0.13	-2.09	3.97	3
GO:0050789 reg. of biological process	reg. of leukocyte cell-cell adhesion (GO:1903037)	↑	-0.48	-2.56	4.40	4
	reg. of mononuclear cell proliferation (GO:0032944)	↑	-1.46	-3.34	7.02	3
	reg. of leukocyte proliferation (GO:0070663)	↑	-1.46	-3.34	7.02	3
GO:0050896 response to stimulus	response to external stimulus (GO:0009605)	↑	-5.59	-8.41	7.67	24
	defense response (GO:0006952)	↑	-10.70	-14.54	12.37	23
	cellular response to chemical stimulus (GO:0070887)	↑	-2.56	-4.73	5.12	20
	response to external biotic stimulus (GO:0043207)	↑	-7.64	-10.78	10.12	19
	response to biotic stimulus (GO:0009607)	↑	-7.62	-10.72	10.07	19
	reg. of response to stress (GO:0080134)	↑	-3.09	-5.36	6.08	14
	reg. of response to external stimulus (GO:0032101)	↑	-3.00	-5.25	6.08	13
	reg. of defense response (GO:0031347)	↑	-4.05	-6.55	7.80	11
	response to cytokine (GO:0SG3097)	↑	-3.50	-5.97	7.38	10
	response to wounding (GO:0009611)	↑	-1.56	-4.05	5.16	10
	inflammatory response (GO:0006954)	↑	-4.19	-6.73	8.67	9
	cellular response to cytokine stimulus (GO:0071345)	↑	-3.14	-5.44	7.03	9
	reg. of response to biotic stimulus (GO:0002831)	↑	-3.21	-5.54	7.49	8
	cytokine-mediated signaling pathway (GO:0019221)	↑	-2.67	-4.85	6.57	8
	wound healing (GO:0042060)	↑	-0.47	-2.54	3.86	6
	response to tumour necrosis factor (GO:0034612)	↑	-1.06	-2.77	5.56	3
	cellular response to tumour necrosis factor (GO:0071356)	↑	-1.06	-2.77	5.56	3
GO:0051179 localization	neurotransmitter secretion (GO:0007269)	↓	-0.47	-2.54	3.86	6
	signal release from synapse (GO:0099643)	↓	-0.47	-2.54	3.86	6
	neurotransmitter transport (GO:0006836)	↓	-0.60	-2.15	3.73	4
	signal release (GO:0023061)	↓	-0.60	-2.15	3.73	4
GO:0065007 biological reg.	reg. of neurotransmitter levels (GO:0001505)	↓	-0.60	-2.15	3.73	4

4.4 Discussion

Understanding the potential ways in which freshwater fish may be able to adapt and change in order to withstand the effects of climate change is vital for informing conservation strategies. In this experiment I aimed to assess for potential routes of thermal adaptation by investigating divergence and plasticity between geothermal and ambient sticklebacks across three population pairs. I tested for evidence of consistent divergence in gene expression, and gene expression plasticity, across three population pairs of sticklebacks inhabiting geothermal-ambient habitat pairs. I found evidence of consistent, robust divergence in gene expression between geothermal and ambient fish across all three population pairs. When considering only the ASHN and the MYV population pairs I found evidence of consistent divergence in gene expression in the brain, in genes relating to a range of gene ontologies.

4.4.1 Divergence between geothermal and ambient sticklebacks

I found evidence of consistent divergence between geothermal in ambient fish for the expression of genes relating to metabolic processes. This is in agreement with previous findings from metabolic phenotypes, as geothermal sticklebacks have a lower metabolic rate relative to their ambient cohorts, (Pilakouta et al., 2020). This divergence is likely an adaptation to the high temperatures of the geothermal habitat, which would typically enforce a higher metabolic rate in resident fish. A higher metabolic rate would increase the calorific needs of resident fish, limiting their ability to invest in growth and reproduction and potentially risking starvation. This selective pressure is expected to be potentially stronger in winter, when invertebrate prey is potentially less available than in summer (Studd et al., 2021). The lower metabolic rate of geothermal fish is therefore expected to allow geothermal sticklebacks to balance their calorific needs in the geothermal habitat (Pilakouta et al., 2020). This hypothesis is further supported by the results of this experiment, as the consistent, robust divergence in metabolic processes found here in the ASHN and MYVN population pairs primarily involves genes relating to glucose metabolic processes. Alterations to glucose metabolism has been documented in other examples of adaptation to nutrient scarce habitats. For example, cave dwelling population pairs of Mexican tetra (*Astyanax mexicanus*) have adapted to the nutrient poor cave habitat through altered glucose metabolism, exhibiting elevated blood glucose levels (Medley et al., 2022;

Riddle et al., 2018). My results suggest that geothermal sticklebacks may also exhibit an adaptation in glucose metabolism in order to reduce starvation risk. I also found an upregulation of genes relating to hypoxia response in geothermal fish. Fish are at a greater risk of hypoxia in warmed habitats due to a combination of reduced oxygen concentration in warmer water (Table 4-1) and an increased metabolism increasing oxygen demand (McBryan et al., 2013; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2005). Indeed, tolerance of higher temperatures has been associated with hypoxia tolerance in fish (Anttila et al., 2013; Firth et al., 2023). My results indicate that geothermal sticklebacks may have adapted a greater tolerance to low oxygen levels and hypoxic events in the geothermal habitat.

Geothermal ASHN and MYV fish were also divergent from ambient fish in the expression of genes relating to protein folding. Protein folding is highly temperature dependent, and molecular chaperones, such as heat-shock proteins, act to prevent the heat-induced denaturation of protein (Feder and Hofmann, 1999). An upregulation in protein-folding related genes has previously been implicated in fish adapted to geothermal habitats (Mahanty et al., 2017; Narum et al., 2013; Oksala et al., 2014). However, I found that protein-folding genes are downregulated in geothermal ASHN and MYV fish when compared to ambient fish. This result may indicate that geothermal sticklebacks are less prone to heat shock than ambient fish. Heat shock proteins are often upregulated in response to acute heat stress (Feder and Hofmann, 1999; Logan and Buckley, 2015; Tomanek, 2010), however, chronic exposure to higher temperatures tends to not to induce an upregulation in heat shock proteins (Logan and Somero, 2010; Podrabsky and Somero, 2004).

A surprising result found in this analysis is that there are a greater number of robust, ambient-geothermal divergent genes in the ASHN population pair than in the MYV population pair in both the brain and liver samples. The larger number of robust, consistently divergent genes in the ASHN population pair is unexpected as this population pair no more than approximately 70 years old, while the MYV population pair is approximately ~2300 years old (Einarsson et al., 2004). Typically, greater divergence would be expected in the older system, as more generations would allow for the gathering of more adaptive or stochastic differences (Ord and Summers, 2015). Both habitats are sympatric, with no

barriers to movement between geothermal and ambient habitats. A greater number of divergent genes in a much younger population pair is therefore unexpected. Notably, this result reflects the results of Pilakouta et al (2020), where geothermal-ambient divergence in metabolism was found to be greater in ASHN fish than in MYV. The heritable difference in morphology between geothermal and ambient MYV sticklebacks was also weaker in the MYV population pair than in other geothermal-ambient pairs (Pilakouta et al., 2023). Strickland et al, (2023) have found extensive gene flow between geothermal and ambient fish at the MYV site, likely limiting genetic divergence in this population pair.

4.4.2 Plasticity divergence within and across population pairs

I found little evidence of consistent ambient-geothermal divergence in plasticity across the three population pairs. However, as the SKR group was particularly affected by the sequencing problems in this experiment, and my previous work, which used a more standard sequencing approach, found a much larger number of plastic genes in this group (see Chapter 3), it is possible that these samples do not accurately represent the population pair.

I predicted that I would find fewer plastic genes in the geothermal fish of each population pair due to the canalisation of ancestral plasticity. I only found support for this prediction in the liver of fish from one population pair. The ASN and SKR groups both showed a much larger number of plastic genes in the brains of geothermal fish than ambient fish, and while I could not compare the plasticity of the MYV geothermal brain to MYV ambient, the MYV geothermal plastic response involves a large number of genes. MYV and SKR geothermal fish livers were also more plastic than their ambient counterparts. This result is counter to my predictions, instead indicating that geothermal fish have consistently become more plastic in brain gene expression. Furthermore, the oldest population pair, MYV, had about twice as many plastic genes in the brain as the much younger ASHN population pair. This result is unexpected, as adaptive plasticity that benefits population pairs colonising novel environments is expected to become canalised through genetic accommodation, resulting in a loss of plasticity (Kelly, 2019). As the MYV geothermal population pair is much older than the ASHN geothermal population pair there has been more time for this loss of plasticity to occur, and so less MYV geothermal plasticity would be

expected. However, this result fits with previous work which found little genetic divergence between MYV geothermal and ambient fish, and yet a large amount of phenotypic divergence, suggesting that that MYV geothermal fish may be particularly plastic (Millet et al., 2013; Strickland et al., 2023). I also found consistency in the genes involved in the brain's plastic response to temperature in MYV and ASHN geothermal fish, potentially suggesting adaptive plasticity. The divergent geothermal plastic response consisted of a widespread upregulation of genes relating to both innate and adaptive immune system functioning at 18^{o}C (Figure 4-3d, Table 4-5).

Warmed aquatic environments tend to have increased pathogen load, diversity and growth rate (Macnab and Barber, 2012; Marcos-López et al., 2010). Furthermore, the interactions between the fish host and its parasite or pathogen are heavily dependent on temperature. For example, the cestode *Schistocephalus solidus* is a common parasite of sticklebacks that benefits from a higher temperature than that preferred by the host (Clarke, 1954; Franke et al., 2019; Macnab and Barber, 2012). As this parasite is known to severely affect the fitness of its host, the mismatch in thermal optima is likely to present a strong selective pressure to fish colonizing the geothermal habitat (Franke et al., 2017; Grecias et al., 2020; Heins and Baker, 2003; Macnab and Barber, 2012). Indeed, the prevalence and abundance of this parasite in sticklebacks has been found to be higher in the geothermal than the ambient habitat of MYV (Karvonen et al., 2013). The prevalence of *S. solidus* is also greater in both geothermal and ambient sourced ASHN fish transplanted to the geothermal habitat of ASHN (see Chapter 2 of this thesis). In my experiment, I found ASHN and MYV geothermal fish to display an upregulation in components of both the active and innate immune system, broadly centring on the activities of lymphocytes and granulocytes. The initial innate immune response to helminths in fish typically involves the mobilisation and activation of granulocytes (Hoole and Arme, 1983; Nie and Hoole, 2000; Sharp et al., 1992; Taylor and Hoole, 1993). Adaptive immunity, involving antibodies against parasite antigens and the proliferation of lymphocytes, is the next line of defence against parasite infections (Rijkers et al., 1980; Roberts et al., 2005; Scharsack et al., 2007). My result potentially indicates an upregulation in these processes in response to an increased temperature, which may provide a protection against *S. solidus* infection. However, previous research has shown little evidence that MYV geothermal

sticklebacks have developed any stronger a resistance to or tolerance of infection by *S. solidus* (Franke et al., 2017, 2019; Karvonen et al., 2013). In fact, MYV geothermal sticklebacks have been found to show lower plasticity in the numbers of head kidney leukocytes in response to *S. solidus* infection than MYV ambient fish (Franke et al., 2017). Potentially, the consistent ASHN-MYV plastic response in genes relating to immune system processes seen in this experiment may represent a plastic response not aimed at *S. solidus* infection. Increased temperatures increase the diversity and growth rate of a variety pathogens, and so the geothermal habitat is likely to present a varied immunological challenge to colonising fish (Macnab and Barber, 2012; Marcos-López et al., 2010). The colonisation of a new habitat also often involves changes to immune system activity, and has been demonstrated in sticklebacks, including genetic changes in neutrophil activity (Beck et al., 2020) and plastic changes in immune gene expression (Stutz et al., 2015). Additionally, the resistance and tolerance of ASHN geothermal and ambient fish to *S. solidus* infection has not been fully tested, and as this population pair was found to exhibit a much greater number of divergently expressed genes between geothermal and ambient fish, perhaps the subtle immune divergence found in the MYV population pair may be stronger in ASHN. My results show that geothermal sticklebacks may have adapted to the geothermal habitat by increasing the activity of the immune system at higher temperatures. However, neither the brain nor the liver are immunologically active organs, and so it is unclear whether this gene expression divergence directly relates to an immunological divergence. Further research concerning the spleen or head kidney would provide an insight into potential immunological divergence.

4.4.3 Limitations of this study

This experiment was limited by the poor survival rate of MYV ambient fish at the 18^{o}C rearing temperature, which resulted in no brains being sampled for this group. This impacted the experimental design and prevented testing for temperature robust geothermal-ambient MYV divergence and for unique MYV geothermal plastic responses in the brain. Furthermore, a large number of samples suffered poor sequencing outcomes, and this disproportionately affected the SKR group, reducing confidence in the results of this experiment.

Additionally, gene expression differences seen between geothermal and ambient sticklebacks may be due to differences at the tissue level, rather than at the regulatory level. As different cells have different transcriptomic profiles, differences in the relative amounts of each type of cell in a tissue may result in an overall divergence in gene expression. As this experiment has no method to determine whether divergent gene expression is due to regulatory or tissue level divergence, further research would be required in order to clarify this divergence. Tissue level divergence would likely be biologically relevant and thus would also reveal potential routes of adaptation and hybrid mismatch.

4.5 Conclusion

Here I found evidence that ASHN and MYV geothermal sticklebacks have diverged from their ambient counterparts in a consistent manner, including both robust divergence in glucose metabolism and hypoxia response, and plastic divergence in immune processes. These results bolster the existing evidence that freshwater fish facing increasing environmental temperatures will need to adapt and change in processes relating to metabolism and oxygen limitation. Our results also indicate that these adaptive changes can occur in as little as 70 generations. Wild fish populations facing climate-change driven increases in temperature may indeed be able to rapidly adapt and persist. However, we found little evidence of adaptive divergence in the SKR population, potentially indicating that, even within the same species, some populations may not show the same propensity for adaptation. Further investigation into the biology of these three populations may reveal the reasons behind this disparity in divergence. Furthermore, my results also highlight the potential importance of plasticity in immune system processes in thermal adaptation. Notably, I found a greater number of plastic genes in the brains of the MYV geothermal fish than in any other group. This population pair also shows little genetic divergence between geothermal and ambient fish, and a high amount of gene flow (Millet et al., 2013; Strickland et al., 2023). These results may indicate that phenotypic plasticity enables thermal adaptation even when genetic adaptation may be hindered by high rates of gene flow. This shows that phenotypic plasticity is an important factor to consider in conservation efforts.

I also find a greater number of divergent and plastic genes in the brain than in the liver, indicating that the brain may be a key organ in thermal adaptation, potentially due to the high energy demands of this organ (Mink et

al., 1981). Further investigation into the anatomy and biology of the geothermal stickleback brain may elucidate the nature of thermal adaptation in the brain.

5 Chapter 5: General discussion

5.1 Summary

Increasing temperatures driven by climate change present a major threat to biodiversity (IPCC, 2021). Animal populations will need to adapt and change in order to persist. Understanding the potential nature of these changes is key to developing effective management plans for the future (Campbell et al., 2017). Evolvability encompasses both adaptive genetic variation and the interactions between genotype, development and the environment (Campbell et al., 2017). Fish are likely to be particularly vulnerable to climate change, as many are ectothermic and there have a limited ability to regulate their internal temperatures (Fry, 1967). Freshwater fish face faster and more extreme increases in temperature than many marine fish, and also often have less options for escape through range shifts (Dudgeon et al., 2006; IPCC, 2013, 2021).

In this project I aimed to take advantage of a unique study system in order to investigate the potential routes of adaptation and adaptive plasticity to novel temperatures in a freshwater fish. Geothermal habitats present a valuable opportunity to study the potential routes of thermal adaptation and plasticity, essentially providing an insight into the future of climate change (O'Gorman et al., 2014; Woodward et al., 2010). Several geothermal-ambient habitat pairs inhabited by three-spined stickleback have been identified in Iceland (Millet et al., 2013; Pilakouta et al., 2020). These habitat pairs provide a "natural experiment" where strong thermal gradients are present over very short spatial distances, minimizing confounding factors such as photoperiod or latitude. Previous work has shown that sticklebacks across these habitats show consistent divergence in morphology and metabolic rate, suggesting that adaptive divergence is occurring (Pilakouta et al., 2020, 2023). In these experiments I aimed to test for evidence of and investigate the mechanisms behind geothermal-ambient stickleback adaptive divergence through complementary field and laboratory-based experiments.

In Chapter 2 I aimed to assess for fitness consequences from transplantation to the alternate habitat. I tested for a relationship between the divergent morphology found in previous work, and proxies of fitness, namely growth and survival. I found evidence for adaptive morphological divergence, as growth (length change) in the non-native habitat was found to relate to head,

posterior and total body shape. Higher growth in fish transplanted to a non-native habitat was found to be associated with shape profiles closer to that of the native fish. The consequences of transplantation were asymmetric with ambient sourced fish transplanted to the geothermal habitat suffering from lower survival rates and greater parasite prevalence than geothermal sourced fish transplanted to the ambient habitat. I also found evidence for divergent shape allometries that related to growth. My findings suggest that wild populations can adapt quickly to thermal conditions. However, immediate transitions to warmer conditions may be particularly difficult.

In Chapter 3 I then used whole transcriptome gene expression to provide a wholly objective insight into the divergence and adaptive plasticity of geothermal and ambient sticklebacks. I focused on an allopatric population pair (SKR) and assessed geothermal and ambient sticklebacks for differential expression and divergent plastic response in gene expression. I then examined the gene expression of hybrids created from geothermal and ambient sticklebacks to provide an insight into the genetic mechanisms behind geothermal-ambient divergence. I found evidence of a small amount of temperature robust divergence in the brain in genes relating to neuron development and functioning. However, much of the divergent gene expression between geothermal and ambient sticklebacks was plastic, and I found little evidence for additive variation or cis-regulatory divergence in hybrid gene expression. These results suggest that evolutionary divergence between geothermal and ambient sticklebacks of this population is not strong and thermal adaptation in this population pair consists largely of plastic shifts.

Finally, in Chapter 4 I examined gene expression patterns across multiple geothermal-ambient stickleback pairs. I assessed for divergence in gene expression consistent across populations. I also assessed for consistent divergence in adaptive plasticity. I found no evidence of consistent divergence across all three population pairs, as the allopatric SKR population pair showed little robust divergence. However, when considering only the ASHN and MYV populations, I found a large number of genes that were consistently divergent between the brains of geothermal and ambient sticklebacks. These genes related most strongly to metabolic processes, suggesting a consistent divergence in metabolism. Furthermore, I found evidence of divergence in plastic responses

in the brain to rearing temperature between geothermal and ambient fish that was consistent across ASHN and MYV populations. Geothermal fish plastically responded to the higher rearing temperature with an upregulation of genes relating to the immune system. Furthermore, I did not find evidence of a consistent divergent response in ambient fish, potentially suggesting that ambient sticklebacks face less selective pressure to be able to respond to an increase in temperature. Notably, I found a greater number of plastic genes in the brains of the geothermal fish in all three populations, and the oldest geothermal population was found to exhibit the most plastic genes.

5.2 Evolvability and plasticity in geothermal sticklebacks

Understanding the ways in which a population may be able to adapt, and change will be key to predicting whether a population will be able to persist under climate change driven temperature increases (Campbell et al., 2017). The concept of evolvability provides a useful framework for understanding the ability of a species to adaptively evolve (Campbell et al., 2017; Feiner et al., 2021; Parsons, 2021). Evolvability describes the capacity of a population to evolve as dependent on its ability to produce *adaptive* variation through genomic, regulatory, and environmental effects (Campbell et al., 2017). Genomic effects can be divided into additive or non-additive effects. Additive effects are present when two alleles contribute equally to phenotype (Falconer and Mackay, 1996). Non-additive effects, include dominance, where one allele contributes more than the other, and epistasis, where there are interactions between genes (Falconer and Mackay, 1996). Regulatory effects can be divided into trans- or cis-regulatory elements (Wittkopp et al., 2004). Cis-regulation is performed by non-coding DNA close to the controlled gene (i.e. promoters, silencers and enhancers) while trans-regulation is occurs via factors distant to the gene (i.e. transcription factors and noncoding RNAs) (Wittkopp et al., 2004; Zhong et al., 2019). Evolutionary divergence tends to be underlain by changes in cis-regulatory processes more often than trans-regulatory changes (Signor and Nuzhdin, 2018; Verta and Jones, 2019; Wittkopp et al., 2004; Zhong et al., 2019). Cis-regulatory variation is more strongly associated with additive loci and are therefore more effectively acted upon by selection (Lemos et al., 2008).

Phenotypic plasticity is able to affect evolutionary processes in a myriad of ways. The rapid, beneficial responses of adaptive plasticity can allow a population to persist long enough for evolutionary change to occur (Baldwin,

1896; Crispo, 2007). Plastic responses may also direct the evolutionary process (Levis and Pfennig, 2016, 2019). The initial plastic response to a novel selective pressure is unlikely to be optimal, and so plastic responses may be further refined and eventually canalized through genetic changes over time (Levis and Pfennig, 2016, 2019). An additional consideration is the ability of phenotypic plasticity to reveal cryptic genetic variation to selection (Parsons et al., 2020).

I show here evidence of adaptive divergence between geothermal and ambient sticklebacks. Ambient sticklebacks were found to suffer from higher mortality rates and greater parasite prevalence when transplanted to the geothermal habitat (Chapter 2), I also found a small number of genes that were divergent between geothermal and ambient sticklebacks independent on rearing temperature, suggesting some degree of potentially genetic divergence (Chapter 2 and 3). However, I found no evidence of a prevalence of additive variation or cis-regulatory divergence when examining gene expression in hybrid fish (Chapter 2). This result was surprising, as I expected to find evidence of genetic divergence between geothermal and ambient sticklebacks in this population pair. Furthermore, I found evidence of divergence in regulatory mechanisms between geothermal and ambient fish as hybrid fish exhibited widespread transgressive expression (Chapter 2). These results may suggest that geothermal and ambient sticklebacks have diverged in trans-regulatory mechanisms. While the evidence for significant genetic divergence between geothermal and ambient sticklebacks is weak, I found evidence of adaptive divergence in phenotypic plasticity between geothermal and ambient fish (Chapter 2 and 3). Additionally, I found evidence of a greater number of plastic genes in the plastic response of geothermal fish in the brains of each population pair (Chapter 2 and 3). The greater plasticity of the geothermal brain is unexpected, as adaptation to a novel habitat is expected to result in a reduction in plasticity over time, as initially plastic responses are refined and canalized into genetic divergence (Levis and Pfennig, 2016, 2019c). This result may suggest that geothermal sticklebacks are still early in evolutionary divergence. However, the MYV population pair, which is estimated to be over 2000 years old, showed the greatest number of plastic genes in the brain of any population pair. This result may indicate that phenotypic plasticity is the main trait under selection in the geothermal habitats, potentially hindering genetic divergence between geothermal and ambient sticklebacks. The high degree of gene flow between

geothermal and ambient sticklebacks in the MYV population (Strickland et al., 2023), may also contribute to selection for plasticity over local adaptation (Sultan and Spencer, 2013).

However, I also found evidence of a potential reproductive barrier between geothermal and ambient fish of the SKR population, in the form of widespread transgressive gene expression in hybrids (Chapter 2). However, it is unknown what effect gene misexpression might have for the fitness of hybrids. Previous work has shown that transgressive or intermediate phenotypes often reduce fitness in hybrids (Pauers et al., 2022). However, in some cases, transgressive phenotypes can lead to improved fitness in hybrids and may even aid the evolutionary process by rapidly generating variation (Dittrich-Reed and Fitzpatrick, 2013; Gallego-Tévar et al., 2019). Additionally, in this experiment we created hybrids between geothermal and ambient fish from an allopatric population pair. It is not known how hybrids between geothermal and ambient fish from sympatric populations may perform. Furthermore, it has recently been shown that there is a high degree of gene flow between geothermal and ambient fish in the MYV population, suggesting that there are few barriers to reproduction in that population pair (Strickland et al., 2023).

5.3 Potential routes of thermal adaptation

5.3.1 Morphology and allometry

Fish inhabiting habitats with increased temperatures have been found to show morphological divergence when compared to ambient populations (Lema et al., 2019; Pilakouta et al., 2023; Rocamontes-Morales et al., 2021; Rowiński et al., 2015). Increased temperatures tend to correlate with a deepening of the body (Georgakopoulou et al., 2007; Lema et al., 2019; Marcil et al., 2006; Pilakouta et al., 2023; Rowiński et al., 2015). Such a consistency in morphological divergence with temperatures suggests that these changes could be adaptive (Pilakouta et al., 2023), and/or influenced by biased developmental responses (Parsons et al., 2020). Tests of the fitness consequences, such as those performed by Ackerly and Ward, (2016) are so far rare. I show here evidence that fish transplanted to the non-native habitat show higher growth when exhibiting a shape profile closer to that of the native fish (Chapter 1). This result provides evidence that the heritable divergence in morphology seen in geothermal and ambient sticklebacks is adaptive.

The divergent morphology of geothermal sticklebacks, as described by Pilakouta et al. (2023), involves a deeper body, a more subterminal mouth and a steeper craniofacial profile. This morphology suggests potentially different foraging and swimming modes relative to ambient sticklebacks (McGee et al., 2013). Specifically, this morphology is in line with a shift to a more benthic lifestyle (Willacker et al., 2010), potentially suggesting a shift away from limnetic prey. This shift may be due to the availability of prey, as geothermal invertebrate communities are typically dominated by large, benthic macroinvertebrates (Nelson et al., 2017; O'Gorman et al., 2012; Scrine et al., 2017). Additionally, this potential dietary shift may also be driven by changes in prey selection. Shifts in feeding strategy occur in geothermal populations of brown trout (*Salmo trutta*) that prefer prey that is higher in the trophic web even when such prey items may be relatively rare (O'Gorman et al., 2016). Furthermore, I found evidence that divergent shape allometries were also related to growth in the transplant experiment. This result may indicate an adaptive role for divergent allometry in geothermal sticklebacks. However, the effect size of this relationship was relatively small and so further research investigating the long-term consequences of allometric divergence in geothermal sticklebacks is needed.

5.3.2 Metabolism

Environmental temperature greatly influences the metabolic rate of fish (Fry, 1967). At the upper end of a fish's thermal tolerance range the energetic cost of basic maintenance is high and the fish will have little scope for activity or growth. Here I show that ASHN and MYV geothermal sticklebacks have diverged in genes relating to hypoxia response, potentially indicating an adaptation to the increased oxygen demands of the geothermal habitat (Chapter 2). Even limited increases in metabolic rate can affect fitness, as energy requirements are increased and an increased food intake is required (Neubauer and Andersen, 2019; Volkoff and Rønnestad, 2020). Geothermal sticklebacks have been found to consistently show a reduced metabolic rate when compared to ambient sticklebacks kept at the same temperature (Pilakouta et al., 2023). Here I show that MYV and ASHN geothermal and ambient sticklebacks are consistently divergent and divergently plastic in genes relating to metabolism (Chapter 2 and 3). A wide range of metabolic processes are affected, suggesting a widespread alteration to metabolism. ASHN and MYV fish were found to be

consistently divergent in genes relating to glucose metabolism (Chapter 2). Divergence in glucose metabolism has also been documented in cave dwelling Mexican tetra, which have inhabited an extremely nutrient poor habitat (Medley et al., 2022; Riddle et al., 2018).

5.3.3 Immune system

Climate change driven increases in temperature are likely to alter the immunological challenges faced by fish. Pathogen load, diversity and growth rate have been found to increase with temperature in aquatic habitats (Macnab and Barber, 2012; Marcos-López et al., 2010). Additionally, the environmental temperature also affects the interactions between parasites and their ectothermic hosts (Franke et al., 2019; Macnab and Barber, 2012). The cestode, *S. solidus*, prefers higher temperatures than its stickleback host (Franke et al., 2019; Macnab and Barber, 2012). This mismatch in thermal optima between host and parasite leads to additional stresses for sticklebacks in warm habitats, as *S. solidus* grows faster at higher temperatures, while the stickleback immune system is impaired (Franke et al., 2019; Macnab and Barber, 2012). In Chapter 1 I show that there is a greater prevalence of this parasite in both geothermal and ambient fish caged in the geothermal habitat than those in the ambient habitat. Additionally, ambient fish caged in the geothermal habitat have a higher *S. solidus* prevalence than geothermal fish in the same habitat. However, as my experiment did not measure other parameters of parasite infection (e.g., parasite mass, parasite load) and also could not assess whether fish were infected prior to or after transplantation, it is not possible to determine whether the decreased prevalence in geothermal fish is a sign of adaptation. However, in chapter 3 I show consistent adaptive plasticity in ASHN and MYV geothermal fish in genes relating to innate and adaptive immune system processes. Several of the immune processes found to be upregulated in geothermal fish at 18^{o}C are known to be involved in the response of fish to helminth parasites (Hoole and Arme, 1983; Nie and Hoole, 2000; Rijkers et al., 1980; Roberts et al., 2005; Scharsack et al., 2007; Sharp et al., 1992; Taylor and Hoole, 1993). However, previous research has found little evidence of adaptation to prevent or resist *S. solidus* infection in geothermal fish in the MYV population (Franke et al., 2017, 2019; Karvonen et al., 2013). As an increased temperature drastically changes the pathogen landscape of a habitat, it is possible that this divergent plastic

response may be in response to alternate pathogen threats (Macnab and Barber, 2012; Marcos-López et al., 2010).

5.3.4 Brain adaptation

In both Chapters 2 and 3 I find a greater number of both divergent and plastic genes in the brain than in the liver. This result may suggest that the brain is a key organ in adaptation to a higher temperature environment. The brain is a metabolically expensive organ, much more so than other organs when at rest (Heldstab et al., 2022; Mink et al., 1981). Temperature and seasonal food availability have both been found to constrain brain size in vertebrates (Gillooly and McCoy, 2014; Luo et al., 2017; Weisbecker et al., 2015; van Woerden et al., 2012). My results show alterations to brain metabolism in geothermal sticklebacks, and in chapter 2 I found that genes relating to neuron function and development were downregulated in the brains of SKR geothermal fish. As such, my results suggest that geothermal sticklebacks may have adapted to higher temperatures by investing less energy into the brain. However, further research is required to fully assess this, as the full impacts of the widespread divergence in genes relating to brain metabolism in ASHN and MYV fish are not clear. Allometric geothermal sticklebacks have been found to be less social than their ambient counterparts (Pilakouta et al., 2022). Sociality is a cognitively taxing activity and so this reduction in sociality may reflect a reduced investment in the brain of geothermal fish.

5.4 Future directions

It is clear that there is still a great deal to be understood about thermal adaptation in freshwater fish. In this thesis I have identified potential routes of thermal adaptation in body morphology and allometry, brain metabolism and neuron development, immune system processes and hypoxia response. I have also shown that phenotypic plasticity play an important role in geothermal stickleback gene expression. Future work might give more consideration to the relationship between thermal habitat and allometric divergence. Whether the allometric divergence observed here is a consequence of the effect of temperature on development, or an adaptive divergence that may reflect differing selective pressures at different points of growth is unknown. Furthermore, there may be an interaction between the two.

Further assessment of the brains of geothermal and ambient stickleback may elucidate the results of this thesis. My results suggest that geothermal sticklebacks may have altered brain metabolisms and potentially reduced investment in neuron development. Previous work has investigated sociality in sticklebacks and identified reduced sociality in an allopatric geothermal stickleback population (Pilakouta et al., 2022). Further research investigating other aspects of cognitive ability in geothermal and ambient sticklebacks may reveal trade-offs in adaptation. If geothermal sticklebacks have reduced cognitive abilities, this may affect their ability to forage successfully in complex habitats (Warburton and Hughes, 2011) and avoid predation (Brown and Braithwaite, 2005). As such, if fish adapting to climate change driven temperature increases show similar reductions in brain investment there may be dire long-term consequences. Reduced cognitive abilities may result in populations of fish becoming more vulnerable to other factors (Fischer and Jungwirth, 2022; Safi et al., 2005).

Brain morphology would also provide an insight into adaptation in the brains of geothermal sticklebacks. As has been noted, vertebrate brain size is constrained by temperature and nutrient availability (Safi et al., 2005). As such, an adaptation in the brains of sticklebacks could involve a reduction in the relative size of the brain. However, the morphological divergence of geothermal sticklebacks involves an increase in the size of the eye relative to the head (Pilakouta et al., 2023). Larger eyes suggests greater investment in the visual senses, which is likely to require greater investment in the brain (Corral-López et al., 2017). As such, geothermal stickleback brains may be divergent in the relative sizes of different regions of the brain. Furthermore, seasonal variation in brain size may be an important consideration. In this thesis I show that the geothermal stickleback brain shows a high degree of plasticity with rearing temperatures. Some vertebrates have been found to show seasonal plasticity in brain size (Burger et al., 2013; Dunlap et al., 2011). As geothermal sticklebacks are expected to experience some additional restriction to prey availability in the winter, it is possible that the brain plasticity observed in this experiment may relate to an adaptive plastic response in the wild.

Further research may also centre on investigating interactions between *S. solidus* and sticklebacks in the natural habitat. While there is previous research

focussing on the interactions between *S. solidus* and stickleback from the MYV population pair (Franke et al., 2017, 2019), my results show that other populations may also be of interest. Reciprocal transplants may be particularly useful for this research, as my results show an increased prevalence in fish in the geothermal habitat, with ambient fish in the geothermal habitat seemingly highly susceptible to *S. solidus*. Further research investigating other parameters of parasite infection in a transplant experiment would provide an insight into the host-parasite dynamics of the geothermal and ambient habitats.

5.5 Conclusions

In summary, the results of my experiments suggest that freshwater fish are able to adapt to increased temperatures within relatively short time periods (~70 generations). Adaptation to the geothermal habitat is complex and appears to involve a wide array of traits, including body morphology and allometry, brain metabolism and neuron development, immune system processes, and hypoxia response. Phenotypic plasticity appears to contribute to thermal adaptation more than genetic divergence, with potential evidence for selection for an adaptively divergent plastic response in geothermal sticklebacks. My results highlight the importance of phenotypic plasticity in thermal adaptation. Populations of fish facing climate change driven increases in temperature may be able to persist through plastic change. However, this is likely to be highly context dependent. Even within a single species I found large differences in the levels of genetic and plastic divergence between geothermal and ambient sticklebacks across populations.

6 Appendix 1: Chapter 2 supplementary materials

Supplementary Table 1: Model design iterations used for AIC model selection. For each analysis the model with the lowest AIC value was selected unless a simpler model had an AIC within 2 of the lowest AIC model. Final selected models are highlighted in blue and bold.

Survival analysis			
Model name	*df*	*AIC*	*Model design*
Model 1	***4***	***182.07***	***Survival ~ source*destination***
Model 2	*12*	*193.27*	*Survival ~ source*destination + cage (nested within destination)*
Model 3	*8*	*181.26*	*Survival ~ source*destination*starting weight*
Model 4	*5*	*183.71*	*Survival ~ source*destination + starting weight*
Model 5	*8*	*181.16*	*Survival ~ source*destination*starting length*
Model 6	*5*	*182.90*	*Survival ~ source*destination + starting length*
Model 7	*6*	*184.26*	*Survival ~ source*destination + starting length + starting weight*
Model 8	*7*	*185.60*	*Survival ~ source*destination + starting length*starting weight*
Model 9	*16*	*193.48*	*Survival ~ source*destination*starting weight + cage (nested within destination)*
Model 10	*13*	*195.27*	*Survival ~ source*destination + starting weight + cage (nested within destination)*
Model 11	*16*	*193.75*	*Survival ~ source*destination*starting length + cage (nested within destination)*
Model 12	*13*	*195.99*	*Survival ~ source*destination + starting length + cage (nested within destination)*
Model 13	*14*	*196.22*	*Survival ~ source*destination + starting length + starting weight + cage (nested within destination)*
Model 14	*15*	*197.54*	*Survival ~ source*destination + starting length*starting weight + cage (nested within destination)*
Model 15	*25*	*215.29*	*Survival ~ source*destination*starting length*starting weight + cage (nested within destination)*
Parasite analysis			
Model name	*df*	*AIC*	*Model design*
Model 1	*4*	*288.04*	*Parasite infection status ~ source*destination*
Model 2	*12*	*299.60*	*Parasite infection status ~ source*destination +cage (nested within destination)*
Model 3	*8*	*292.96*	*Parasite infection status ~ source*destination*starting weight*
Model 4	*5*	*287.88*	*Parasite infection status ~ source*destination + starting weight*
Model 5	*8*	*289.07*	*Parasite infection status ~ source*destination*starting length*

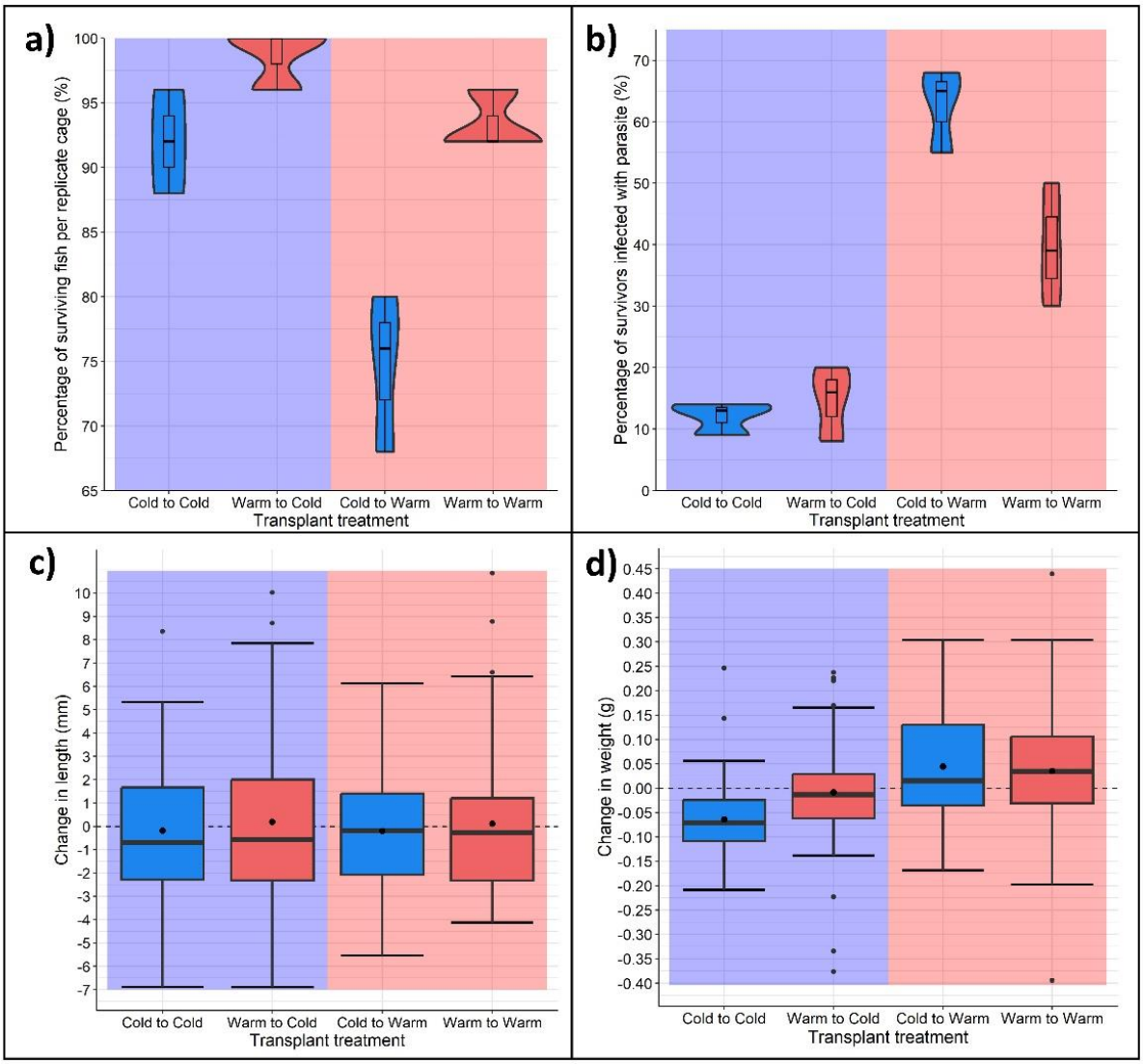
Model 6	*5*	*286.41*	*Parasite infection status ~ source*destination + starting length*
Model 7	*16*	*305.40*	*Parasite infection status ~ source*destination*starting weight +cage (nested within destination)*
Model 8	*13*	*300.03*	*Parasite infection status ~ source*destination + starting weight +cage (nested within destination)*
Model 9	*13*	*297.19*	*Parasite infection status ~ source*destination + starting length +cage (nested within destination)*
Model 10	*16*	*299.94*	*Parasite infection status ~ source*destination*starting length +cage (nested within destination)*
Model 11	*16*	*297.62*	*Parasite infection status ~ source*destination*starting weight*starting length*
Model 12	***7***	***282.40***	***Parasite infection status ~ source*destination + starting weight*starting length***
Model 13	*15*	*294.62*	*Parasite infection status ~ source*destination + starting weight*starting length +cage (nested within destination)*
Model 14	*24*	*309.95*	*Parasite infection status ~ source*destination*starting weight*starting length +cage (nested within destination)*
Model 15	*14*	*297.66*	*Parasite infection status ~ source*destination + starting weight + starting length +cage (nested within destination)*

Weight change analysis

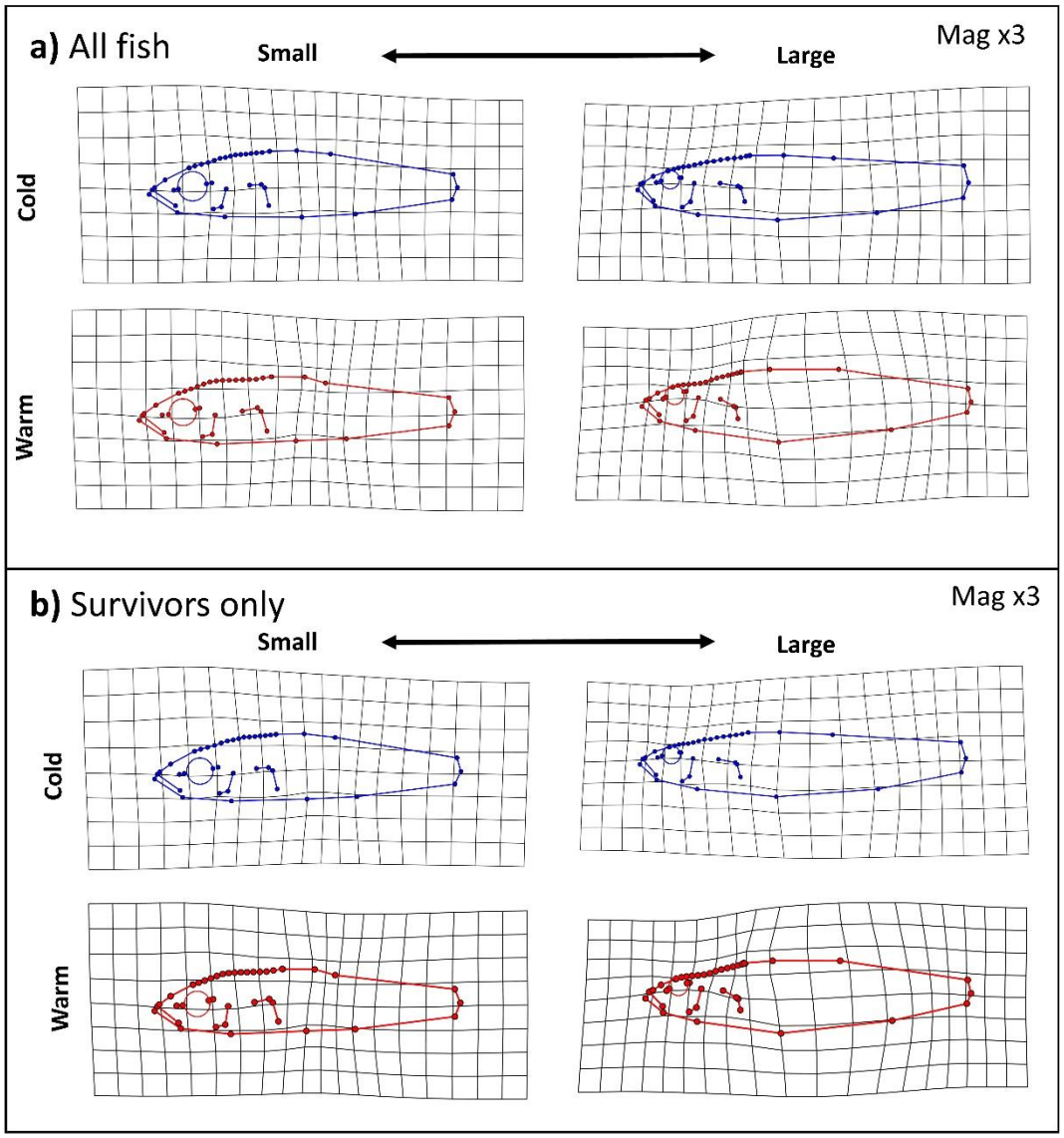
Model name	***df***	***AIC***	***Model design***
Model 1	*5*	*-444.44*	*Residual weight change ~ source*destination*
Model 2	*13*	*-462.78*	*Residual weight change ~ source*destination + cage (nested within destination)*
Model 3	*17*	*-480.42*	*Residual weight change ~ source*destination*Parasite infection status + cage (nested within destination)*
Model 4	***14***	***-486.42***	***Residual weight change ~ source*destination + cage (nested within destination) + Parasite infection status***
Model 5	*9*	*-460.01*	*Residual weight change ~ source*destination*Parasite infection status*
Model 6	*6*	*-465.85*	*Residual weight change ~ source*destination + Parasite infection status*

Length change analysis

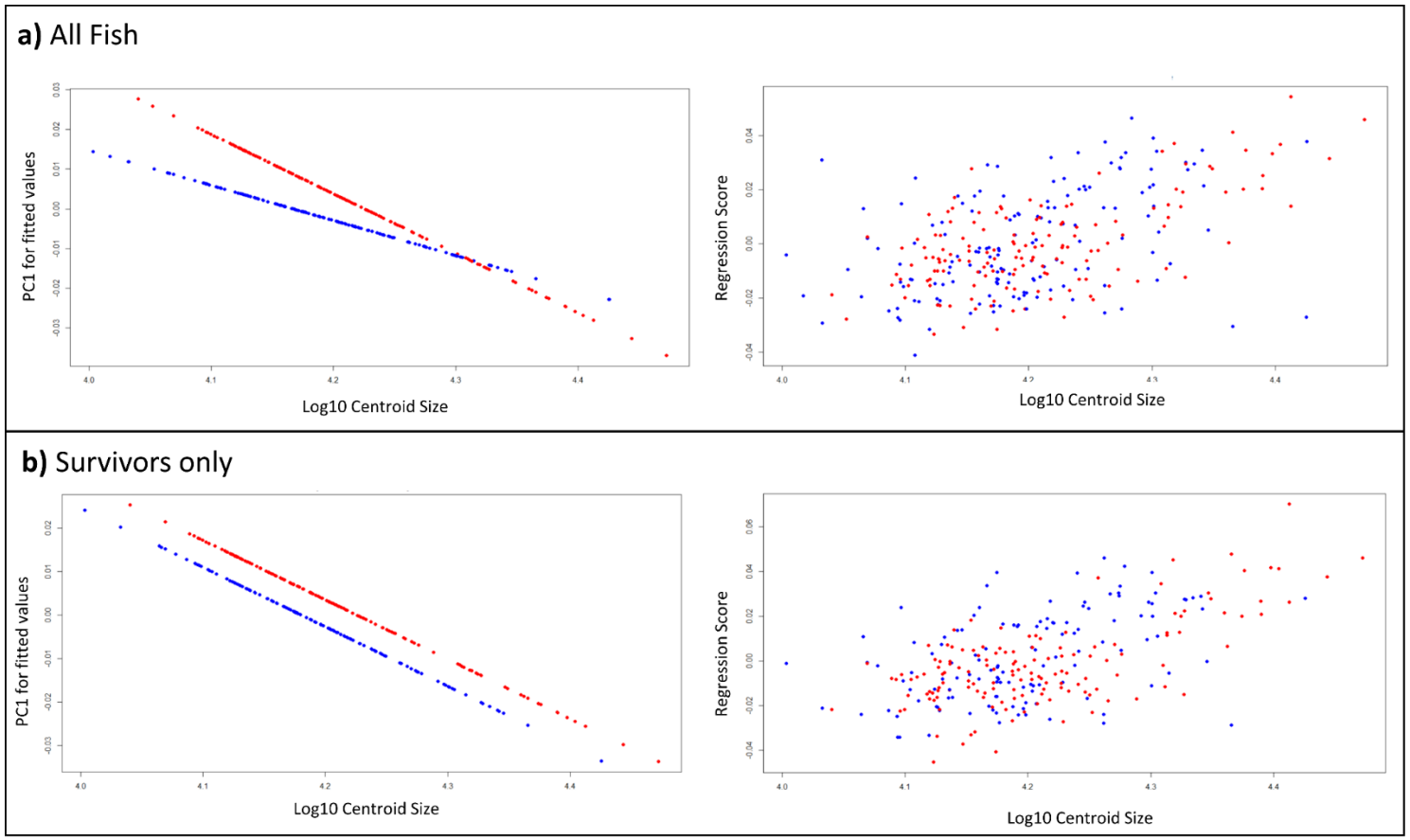
Model name	***df***	***AIC***	***Model design***
Model 1	*5*	*1379.12*	*Residual length change ~ source*destination*
Model 2	***13***	***1364.98***	***Residual length change ~ source*destination + cage (nested within destination)***
Model 3	*14*	*1366.88*	*Residual length change ~ source*destination + cage (nested within destination) + Parasite infection status*
Model 4	*6*	*1380.87*	*Residual length change ~ source*destination + Parasite infection status*
Model 5	*9*	*1385.21*	*Residual length change ~ source*destination*Parasite infection status*
Model 6	*17*	*1371.77*	*Residual length change ~ source*destination*Parasite infection status + cage (nested within destination)*



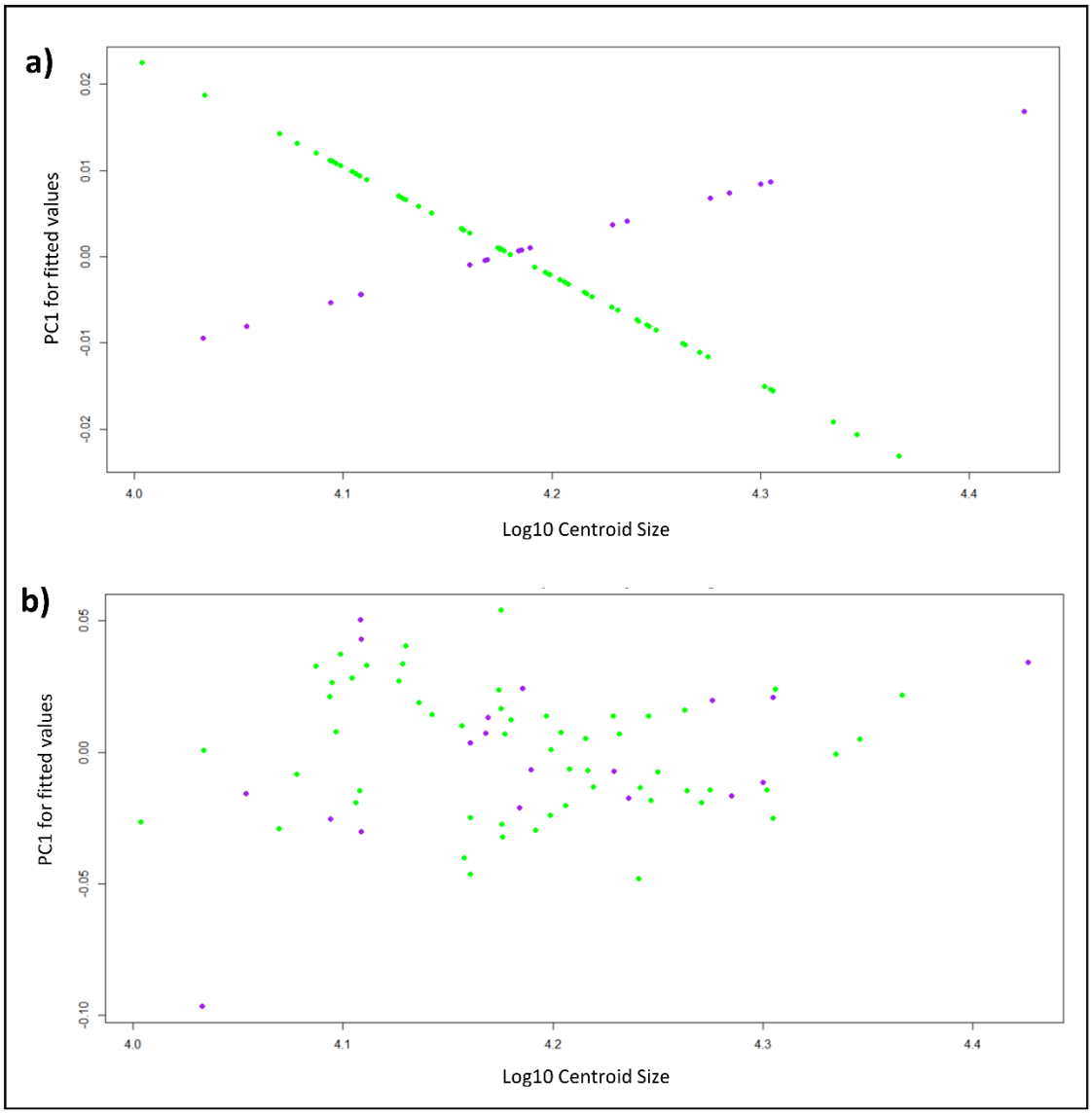
Supplementary Figure 1 Violin and box and whisker plots displaying variation in a) the mean percentage of surviving fish per cage per treatment, b) the percentage of surviving fish found to be infected with S. solidus, c) the residual change in length and d) residual weight changes (g) adjusted for parasite and cage effects, that occurred with experimental treatments in the reciprocal transplant experiment involving natural cold and warm habitats. Top and bottom hinges represent 25th and 75th percentile, centreline represents 50th. Black dot displays mean residual weight change. Whiskers give 95% confidence interval. Blue and red filled boxes represent the cold and warm sourced fish respectively, blue and red backgrounds represent the cold and warm destination habitats, respectively.



Supplementary figure 2 Deformation grids (with 3x magnification) depicting the allometric shape variation (with centroid size as size) of warm and cold source fish for datasets of a) all fish used in this experiment and b) surviving fish only. Here relationship between centroid size and shape are plotted to the extremes of size.



Supplementary figure 3. Plots depicting allometric relationships for warm (red) and cold (blue) source fish for datasets of a) all fish used in this experiment and b) surviving fish only. Left hand plots show first principal component of the "predicted" values versus size from calculated fitted values from procD.lm selected by test of allometry type (All fish – unique allometry between sources, Survivors only – common allometry). Right hand plots show calculated standardised shape scores from the regression of shape on size plotted against size based on test of allometry type type (All fish – unique allometry between sources, Survivors only – common allometry).



Supplementary figure 4. Plots depicting allometric relationships for surviving (green) and dead (purple) within the cold to warm treatment group. Plot a) shows first principal component of the "predicted" values versus size from calculated fitted values from procD.lm selected by test of allometry type (unique allometry between dead and surviving fish). Plot b) shows calculated standardised shape scores from the regression of shape on size plotted against size based on test of allometry type type (unique allometry).

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