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From genotype to phenotype: Characterising intraspecific variation in the three-spined stickleback (*Gasterosteus aculeatus*) in the Burrishoole aquatic ecosystem complex in western Ireland

Thesis presented by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, at either University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Floriane Leseur

Thesis summary

The stickleback *Gasterosteus aculeatus* is a resident of coastal marine and freshwaters throughout the temperate Holarctic and is a model species for evolutionary biology, particularly due to its recolonization events of freshwaters in previously glaciated catchments. This study concentrates on the species in Ireland, particularly in the Burrishoole system in the west coast of Ireland. The latter is an extensively studied catchment with freshwater streams and lakes running into a brackish lake over waterfalls largely impassable upstream to sticklebacks; then connecting to coastal waters. Previous genetic (using microsatellites), morphological and meristic studies in the Burrishoole catchment identified four statistically distinct populations; one in the major freshwater lake and three (completely-, partially- and low-plated forms) in the brackish lake. Here, a more extensive study was undertaken extending the geographic and temporal analyses of genetic aspects (using neutral and adaptive SNPs) and phenotypic traits including morphological and metabolic characters, and gut microbiome composition. Neutral SNPs showed largely the same population composition as previously suggested using microsatellites (i.e. isolation-by-adaptation reflective of time of divergence), though the partially-plated group in the brackish lake was not supported. This composition being temporarily stable and supported by morphological analysis. The confirmed adaptive SNPs suggest a different scenario with isolation-by-environment in relation to the two populations differentiated by ecomorph (plates, body shape, gill rakers and migratory behaviour) co-existing in Lough Furnace. Analysis of traits associated with metabolism and gut microbiome did not extend population differentiation, but yielded novel results individually. The individual results are discussed in four separate chapters, and the thesis then concludes with an overview chapter. Similarity or differences with other stickleback

studies around the North Atlantic and Pacific are explored, and possible isolating mechanisms between the completely- and low-plated forms co-occurring during the spawning season in the brackish lake identified (migratory behaviour – anadromy - in the completely-plated form, absence of evidence of hybridisation in co-habitation, wash out from the freshwater population into the brackish lake). The rare physical characteristics observed in the Burrishoole system may allow clarification of the process of recolonization of freshwaters in this species.

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Thesis structure

Each data chapter in this thesis is intended ultimately to be submitted for publication in peer-reviewed journals and therefore, while as thesis chapters are expansive and more detailed than a paper for publication, as far is possible, are still formatted and presented as stand-alone manuscripts. To facilitate the reading, all figures and tables are located within the text rather than being at the end of each chapter. Chapters are cross-referenced when appropriate, but can be read as a separate document. A citation list is included at the end of each chapter. At the beginning of each chapter, I have indicated if chapters have already been submitted or in preparation.

I acknowledge here the various contributions to the data chapters in my thesis:

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Contributions to chapter 2: FL wrote the chapter and performed the analysis. FL, TR, JK and PMcG conceived the study. FL, KP, SR, ER, CW, RG, RP and JK collected the data.

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Contributions to chapter 4 (in review as paper for publication in the *Journal of Fish Biology*): F.L. performed the analysis and wrote the original manuscript, with the support of K.P. F.L., K.P., J.K., S.R., C.W., L.R., R.G. collected the samples and/or performed the experiment. F.L, K.P and P.MG. conceived the study. P.MG., E.J. and P.P. obtained the funding. All authors have approved the final manuscript.

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Chapter 1 : General Introduction

Intraspecific variation, the variation that exists within species, represents a large and important and often under-appreciated component of the diversity of life on Earth and is discernible across a wide range of continuous and discrete morphological, behavioural life-history traits (Roff, 1996). This variation underpins a species' or a population's evolutionary potential (Haig *et al.*, 2006) and is a significant consideration in determining the nature of adaptations to different environments and the capacity to respond to environmental change e.g. global warming. However, it can be challenging to understand the sources of this variation and the factors that sustain it as genotype, environment, and genotype by environment interactions (phenotypic plasticity within a specific environment) all play roles that contribute to promoting and maintaining population differences (DeWitt & Scheiner, 2004).

Intraspecific variation can involve differences between populations and differences within populations. Intraspecific variation can also be characterised in terms of continuous variation versus discrete variation. Distinct morphotypes or behavioural phenotypes, for example, sometimes co-occur within the same environment or geographical region. These discrete types may or may not be reproductively isolated (or be partially isolated) from each other. For example, distinct migratory types of salmonid fishes can occur in the same population (Chapman *et al.*, 2012; Dodson *et al.*, 2013), but freely interbreed. In such situations there can still be a heritable component to this discrete variation as well as environmental influences. However, in other situations there maybe partial reproductive isolation between sympatric or parapatric types, which may be a precursor to full speciation. Therefore, discrete intraspecific variation can comprise

a continuum spanning from within population variation to distinct population segments (termed evolutionarily significant units in the USA for the proposes of the Endangered Species Act (ESA) (Waples, 1995)) to races or sub-species. As Ginsburg (1937) long ago pointed out "The question of what is a species, or a sub- species, or a race, or any classification of specific or lower rank category, cannot be disassociated from one another".

An abundance of terms exist to describe at the population level discrete intraspecific variation such as 'morphotype', 'ecomorph', 'ecotype', 'species pairs', 'ecomorphotype', 'ecomorph pairs', 'epitype' and 'life-history' types (Hufford & Mazer, 2003; Clemens & Schreck, 2021). These descriptions are often used synonymously or complementarily, depending on the context. In the case of ecomorphs (distinct morphological types), it is difficult to determine to what extent they are reproductively isolated, genetically differentiated at neutral versus adaptive loci, behaviourally different, physiologically different, and divergent in terms of life histories. This thesis explores these fundamental questions using stickleback as a classic model system.

I use the term ecomorph throughout this thesis to simply mean different morphological types within a species regardless of whether there is a genetic basis to these phenotypic differences or regardless of the extent of reproductive isolation, like used by Wootton (2009). For example, note however, some authors use the term ecotype assuming a genetic basis and to some extent reproductive isolation. However, the term ecomorph fundamentally describes the typical phenotype of the individual in a population, which would be for example, the combination of morphological, physiological, migratory, and immunological traits…etc. Similarly, the ecotype would be the combined genetic characterisation of a population.

Studying ecomorphs within a system is of considerable interest as a basis for understanding the nature of those evolutionary processes contributing to their development and maintenance, as they are likely to be the first step of a continuum of stages towards eventual speciation (Thorpe *et al.*, 2005). Speciation may arise if the divergence in key traits, such as morphological, physiological or behavioural, is strong enough to lead to "ecological speciation" and reproductive isolation. Ecological speciation is the result of natural selection driving divergence between different populations, and as a consequence giving rise ultimately to reproductive isolation (Mayr, 1947; Rundle & Nosil, 2005; Nosil, 2012; Faria *et al.*, 2014). This process is possible in allopatry (geographically isolated populations in different areas), parapatry (populations that partially overlap in an area), or sympatry (populations that overlap in the same area). The maintenance of reproductive isolation between these nascent populations can then lead to the development of genetically distinct demes, which, in this context, might develop into distinct ecomorphs. However, it can be difficult to find evidence for the progression of ecological speciation processes ongoing in a system, as reproductive barriers do not necessarily develop in tandem with adaptive divergence (Hendry, 2009). The decrease of gene flow between populations, whether due to stochastic processes such as genetic drift (Lande, 1981), or to deterministic process such as natural selection (Endler, 1986) or the reinforcement of reproductive barriers due to selection against hybrids (Coyne & Orr, 2004), can potentially result in complete reproductive isolation and, ultimately, species formation. Ecological speciation is not necessarily discrete, and can be a continuum with different divergence levels and stages of adaptive divergence (Butlin *et al.*, 2008; Hendry, 2009; Hendry *et al.*, 2009; Theis *et al.*, 2014).

Studying intraspecific variation also presents the opportunity to obtain a better understanding the processes such as local adaptation and of parallel evolution. Parallel

evolution can be defined as “evolution of two (or more) populations in very similar directions in trait space” (all the different forms/outcomes a trait could take) (Bolnick *et al.*, 2018), but the term and thus the process are disputed, with some authors arguing that “convergence” should apply only to the comparison of phenotypes and “parallel” should imply genes (Scotland, 2011; Rosenblum *et al.*, 2014). It has also been suggested that the outcomes of parallel evolution should be described as a continuum, using quantitative measures of the trait of interest, instead of considering it as a binary phenomenon (parallel or non-parallel) (Oke *et al.*, 2017; Speed & Arbuckle, 2017; Stuart *et al.*, 2017; Langerhans, 2018). Establishing how a given trait has evolved in different populations under similar abiotic and possibly biotic and/or associated biotic constraints, but in different systems, is a good place to start in order to compare and study parallel evolution.

Ecomorphs (based on morphological attributes) / ecotypes (defined both morphologically and genetically), and their differentiating characteristics, have been studied in diverse taxa including plants, fish, mammals (Hufford & Mazer, 2003; de Bruyn *et al.*, 2013; Clemens & Schreck, 2021). In marine mammals, among killer whales for example, five major ecotypes have been characterized based on different phenotypical traits such as their morphology, diet, behaviour and distribution across the Northern and Southern Hemispheres (de Bruyn *et al.*, 2013). Interestingly, two populations in partial sympatry (“resident” and “transient” forms) distributed in the North Pacific, specialising on different prey (fish or marine mammals), were found to be more genetically distinct from each other than resident ecotypes found in the North Atlantic. Similarly, another study on *Orca orchinus* observed that the resident ecotype found in the North Pacific and the resident ecotype from the North Atlantic were less alike genetically compared to the transient ecotype from North Atlantic, suggesting, that while quite different genetically, they nevertheless had similar ecological specializations, was evidence of parallelism or

possibly a shared phylogenetic history (Filatova *et al.*, 2015). All these differences contributed to causing and subsequently maintaining reproductive isolation between these sympatric ecotypes, that have undergone strong founder effects from the ancestral colonies, and genetic drift during the last glaciation maximum (LGM) (Foote *et al.*, 2019). Therefore, the repeated occurrence of similar ecomorphs in independent populations gives the opportunity to study adaptation as a result of parallel evolution.

Stickleback: a complex species

The three-spined stickleback (*Gasterosteus aculaetus*, L. 1758, henceforth, stickleback) is a small teleost fish species from the family Gasterosteidae. It has become an important model species deployed in a variety of different research areas including evolutionary biology and ecology, physiology, microbiology with respect to the characterisation of microbiome communities, sexual selection and host-parasite co-evolution (Ostlund-Nilsson *et al.*, 2006; Huntingford & Ruiz-Gomez, 2009; Barber & Nettleship, 2010; Barber, 2013). The species is distributed widely across the Holarctic (Wootton, 1976; Bell & Foster, 1994), and found in a variety of contrasting environments, such as along salinity gradients from purely freshwater to marine environments and also in acidic and hypoxic conditions. Stickleback are often referred to as a species complex due to the remarkable level of phenotypic and ecological diversity found present within a single species (McKinnon & Rundle, 2002). Importantly, the stickleback's value as a model species and its usefulness for examining evolutionary processes, stems from the phenomenon that contemporary freshwater populations have independently and repeatedly recolonised freshwater environments from marine ancestral populations (Bell & Foster, 1994; McKinnon & Rundle, 2002). This has resulted in an adaptive radiation

of ecomorphs in multiple different situations and hence providing an excellent opportunity to study parallel evolution. These features have made the stickleback a classic for studies of divergent selection resulting in adaptive variation, the emergence of reproduction isolation as a result of or independent of any physical barriers, which ultimately can become complete and irreversible and how this can contribute to the concept of the species continuum (Hendry *et al.*, 2009). The multiple ecomorphs pairs (marine-freshwater, lake-streams, limnetic-benthic, mud-lava) that have been observed in different systems offers exceptional insights to study parallelism. In addition to being an excellent model for the study of evolutionary and ecological processes in the wild, the stickleback can be easily maintained and manipulated in both laboratory and field experiments.

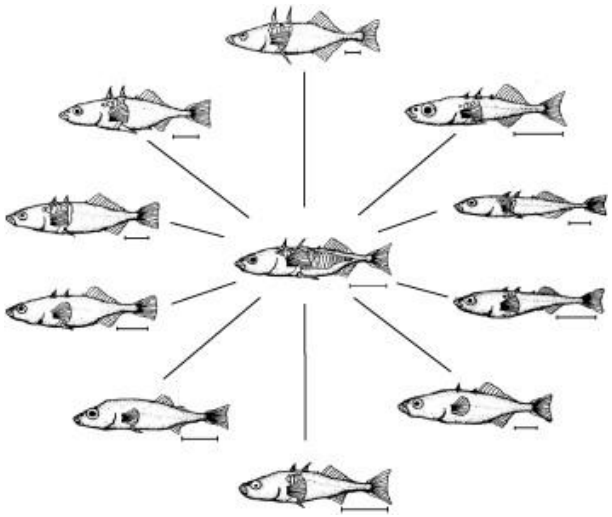

Figure 1.1. Adaptive radiation in the three-spined stickleback. The central stickleback represents the marine lineage (ancestors) and all the others represent different brackish and freshwater populations with their phenotypical characteristics. Taken from Bell MA and Foster SA (eds.) (1994) *The Evolutionary Biology of the Threespine Stickleback*. Oxford: Oxford University Press.

One of the most studied phenotypical characteristic is the partial or complete loss of lateral plates in freshwater populations (Bell *et al.*, 2004; Barrett *et al.*, 2008). Two main hypotheses have been proposed for plate loss: firstly, their potential role against predators (Reimchen, 2000) or secondly, the osmoregulation change in freshwater environments (due to the decrease of calcium in freshwater) (Heuts, 1947; Giles, 1983). Based on the number of lateral plates, different ecomorphs/ ecotypes or (sub)species like the *Gasterosteus nipponicus* (Higuchi *et al.*, 2014) have been proposed (Münzing, 1963; Hagen & Gilbertson, 1973) such as the completely-plated, potentially anadromous morphotypes, observed in marine and brackish environments; the partially-plated and resident morphotypes found in brackish environments; the low-plated, resident in freshwater and brackish environments. Lateral plating is not the only phenotypic trait that may play a role in protecting the fish against predators; as its common name suggests, stickleback have three dorsal spines, as well as two pelvic spines as an additional defence mechanism. Some variation exists in the number of dorsal spines in different populations (Reimchen, 1980; Zanella *et al.*, 2009), in addition to the absence of pelvic spines in some freshwater populations (Colosimo *et al.*, 2004; Chan *et al.*, 2010). Also, more generally stickleback body shape is associated with the ecology of the populations and is considered an important adaptive trait (Reid & Peichel, 2010), in respect of swimming performance (Hendry *et al.*, 2011) and foraging behaviour (Robinson, 2000).

In addition to morphological comparisons molecular methods have also been deployed to reveal the genetic basis of divergent traits in stickleback populations. For example, a genome-wide linkage map study revealed that several quantitative trait loci (QTL) play a role in lateral plate number, dorsal spine number and gill raker length (Peichel *et al.*, 2001). In other studies focused on identifying the genomic regions responsible for the partial or complete loss of lateral plates, the best characterised is the mutation in the

Ectodysplasin gene (*EDA*) that predicts plate loss (Colosimo, 2005; Defaveri *et al.*, 2011; Shimada *et al.*, 2011b). Other genes identified as being partially responsible for variation in other phenotypic traits in sticklebacks include a deletion in the Pitx1 gene, causing the loss of pelvic spines in certain freshwater populations (Shapiro *et al.*, 2006); a change in the regulatory sequence in the Kitlg gene responsible for pigmentation divergence (variation in skin and gill colours) (Miller *et al.*, 2010); and, more recently, a mutation in the Fads2 gene complex that causes a fatty acid deficiency that may contribute to freshwater adaptation (Ishikawa *et al.*, 2019).

These morphological and ecological differences, supported by genetic studies, reveal and underscore the complexity of intraspecific variation in co-existing stickleback ecomorphs, in allopatry, parapatry or sympatry. The presence and the strength of reproductive barriers between different ecomorphs has been found to be different across systems (Hendry *et al.*, 2009). Several studies have focused on characterising the nature of these barriers. In benthic-limnetic ecomorph pairs in Canada, assortative mating (Raeymaekers *et al.*, 2010; Räsänen & Hendry, 2014) and allochrony (Hanson *et al.*, 2016a) were absent, whereas in another stream-lake ecomorph pairs, these two reproductive barriers were identified as playing an important role in reproductive isolation (Eizaguirre *et al.*, 2011; Andreou *et al.*, 2017). In a marine anadromous-freshwater pair living in sympatry or parapatry in a Scottish tidal estuary, Jones *et al.* (2006) found little evidence for gene flow between them, suggesting strong selection against hybrids.

Sticklebacks have also been used as a model to study life-history traits and environmental factors that influence metabolic traits. The different colonization events of brackish and freshwater environments and subsequent associated ecomorph diversity presents an opportunity to study how metabolic rate may have evolved in different freshwater and

marine environments. For example, some studies have focused on studying marine anadromous and freshwater resident ecomorphs, observing that freshwater residents showed a reduced capacity for prolonged swimming compared to marine individuals (Taylor & McPhail, 1986; Tudorache *et al.*, 2007). Other studies have focused on the impact of abiotic factors such as temperature (Ressel et al., 2021) and salinity (Grøtan *et al.*, 2012) on the potential divergence of metabolic rate. In wild populations in Iceland, individuals residing in a warm lake environment had a lower standard metabolic rate SMR than individuals residing in colder lake environment (Pilakouta *et al.*, 2020). Stickleback microbiome communities have also been investigated in different ecomorphs, and have shown repeated and parallel shifts among the different ecomorphs, similarly to shifts in phenotypic traits among ecomorphs (Rennison *et al.*, 2019). Further studies have shown the importance of environmental factors in directly shaping the microbiome community diversity through diet (Bolnick *et al.*, 2014c) or indirectly, whereby populations occupying different niches can via their immune system response to microbes encountered in the environment, shape their microbiome through a process of microbial community filtering. In addition to underpinning immunological responses to the environment and given that sex is genetically determined in sticklebacks, genotype also plays a significant role through sex in shaping microbiome community composition, for example, female and male stickleback have been found to respond very differently to diet manipulation (Bolnick *et al.*, 2014b). Host immune system traits were also strong predictors of gut microbiome (Bolnick *et al.*, 2014a). Therefore, studying metabolic traits and microbiome community composition among different ecomorphs overlapping in the same geographic location would provide an opportunity to highlight and quantify intraspecific variation, as well as the mechanisms that may contribute to maintain this variation.

Sticklebacks life-history in Ireland and in the Burrishoole system

In Ireland, various different scenarios of colonisation have been suggested to have occurred during the late Pleistocene period following the last maximum glaciation (LGM). One hypothesis suggests that Ireland might have been close to a cryptic refugium (a refugium where conditions considerably differ from the surroundings areas), during the period, and was one of the first regions to be colonised by anadromous species after the retreat of the ice sheet, for example on *Salmo trutta* (McKeown et al., 2010) and *Gammarus duebeni* (Krebes *et al.*, 2010). It has also suggested that this refugium played a role in the freshwater colonisation by marine sticklebacks. A study of mitochondrial and microsatellites markers showed that two major lineages, a Trans-Atlantic (TA) lineage and a European (EU) lineage, have colonised Ireland on multiple occasions, in response to sea level oscillations (Ravinet *et al.*, 2014). Ravinet *et al.* (2014) also identified a third haplotype, which they reported was unique to Ireland (not observed in other parts of Europe, like TA and EU), and was consistent with the hypothesis that Ireland could have been a cryptic refugium. Another study showed that sticklebacks have colonised many systems in Ireland and strong parallelism has been identified between each ecomorph pairs (limnetic-benthic or marine-freshwater), as these ecomorphs followed the same direction and were very similar (Ravinet *et al.*, 2013).

The stickleback in the Burrishoole system, particularly those found in Lough Furnace, have received considerable amount of study recently (Ravinet *et al.*, 2015) and are the focus of my research here. The Burrishoole catchment is located in County Mayo in western Ireland (N53°55′22″, W9°34′20″). This catchment is composed of a network of rivers connected to the freshwater Lough Feeagh, the waters of which flow in sequence

to the brackish Lough Furnace and then directly to the Atlantic Ocean via Clew Bay (Figure 1.2).

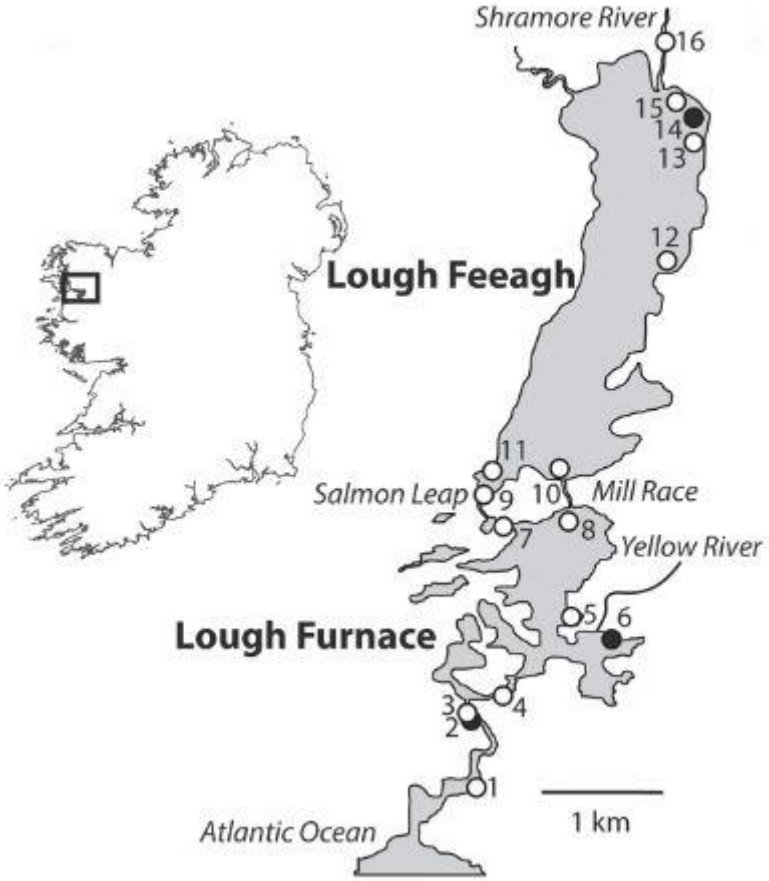

Figure 1.2. Map of the Burrishoole catchment and its location on the west coast of Ireland, with Lough Feeagh and Lough Furnace. This figure is extracted from Ravinet *et al.*, (2015). Black circles indicate sites sampled in 2009, white circles indicate sites sampled in 2010. An additional site, Shrarevagh River is not shown on this map but lies 4 km north of Shramore River.

Lough Feeagh is oligotrophic, with an area of 410 ha, a mean depth of 14.5 m, and a maximum depth of 46 m (de Eyto *et al.*, 2016), with two main inflows, the Glenamong and Black River. Lough Feeagh is classified as a dystrophic lake because of the low nutrient concentrations and low primary productivity (Ryder, 2015). The lake is usually

stratified in summer, influenced by rainfalls and floods. However, strong and rare weather events, like Storm Hector in spring 2018, can cause sudden and complete mixing events in the lake (Calderó-Pascual *et al.*, 2020). Lough Feeagh is connected to Lough Furnace by two channels: the natural Salmon Leap and the artificial Mill Race.

Lough Furnace is a deep and stratified tidal lagoon characterised by a complex shape connected to both purely freshwater and marine environments (Cassina, 2012; Cassina *et al.*, 2013). Unusually for such a body of water in temperature latitudes, it is permanently stratified. Two stable and stratified salinity layers occur between the surface and deeper water that are maintained by a strong and shallow pycnocline (~2.4 m). Lough Furnace is also characterised by high concentrations of dissolved oxygen at the surface and virtually anoxic conditions below 6-7 m depth. In case of large scale, but relatively rare extreme weather events, the shallow and deep layers can mix rapidly (in a single day), leading to radical, lake-wide change in temperature and oxygen (Kelly *et al.*, 2018a,b). Lough Furnace has a strong, north-south, salinity gradient with low salinities in the north of the lake (2-4 PSU) graduating to brackish salinities near the channel that connects to Clew Bay (10-15 PSU). However, below the pycnocline, the salinity is much higher even in the north of the lake (~22 PSU).

Muingdoran, the other main location considered in this thesis is located approximatively 50 km up north from the Burrishoole catchment (N54°10′30″, W9°99′04″). Along the shoreline, many rock pools are formed and refreshed by the tide cycles. The conditions in the rock pools vary a lot from one rock pool to another with salinities between 22 to 36 PSU. The rock pools are connected to the Atlantic Ocean at high tides, but separated at low tides.

A previous study of sticklebacks in the Burrishoole system has identified three overlapping populations that are different genetically and morphologically: a low-plated

freshwater resident in Lough Feeagh, a low-plated brackish resident and a completely-plated potentially anadromous form in Lough Furnace (Ravinet *et al.*, 2015). In Lough Furnace, this study also proposed a third ecomorph, a partially-plated one, which was suggested to comprise hybrids between the low and completely-plated ecomorphs. However, there is little evidence for hybridisation and that reproductive isolation between the low and completely-plated ecomorphs might be important (Ravinet *et al.*, 2015). Their study was based on nine microsatellites in addition to five other loci that are putatively linked to Quantitative Traits Loci (QTL) for EDA. These markers differentiated ecomorphs in the Burrishoole system genetically, but were equivocal in respect of the partially-plated ecomorph and whether these were a distinct ecomorph or the result of hybridisation between complete plated and low plated individuals. Even if reproductive isolation was highly likely as the most plausible explanation, Ravinet et al. (2015) recommended future studies to determine whether potential reproductive barriers were occurring in this system, and to better characterize phenotypic differences among the ecomorphs and which abiotic factors could be influencing the level of adaptive divergence observed.

Objectives and overview of the thesis

The overall objective of this thesis is to explore the intraspecific variation of different stickleback populations by studying diverse morphological, phenotypical and genetic traits, and ascertain (indirectly considering the results of analysis of neutral and adaptive genetic markers) what abiotic factors might promote the development of such variation.

In chapter 2, I define and describe some of the morphological characteristics of different ecomorphs/ecotypes as previously reported by Ravinet *et al*. (2015) found in the Burrishoole system through an extensive spatial (17 sites) and temporal (three years)

sampling regime. I studied the different metric, meristic and morphological traits that characterise these ecomorphs. I also investigated which reproductive barriers might play a role between two brackish stickleback ecomorphs (one low-plated and one-completely plated) through a semi-controlled breeding experiment.

In chapter 3, I characterised the population genetic structure associated with the different stickleback ecomorphs in the Burrishoole system as proposed by Ravinet *et al*. (2015) using microsatellites and subsequently revised by me in Chapter 2 using SNP markers, including two geographically proximate wholly marine populations as a reference. I further characterised patterns of neutral and adaptive divergence grouped *a priori* based on the ecomorphs identified in Chapter 2 using two classes of single nucleotide polymorphism (SNP) markers obtained from the stickleback literature to distinguish between the effects of neutral and adaptive processes and their contribution to population genetic structure and adaptive divergence.

In chapter 4, I investigated, using respirometry set-up, the differences in metabolic traits (SMR, MMR and AS) of the different populations and assess how biotic (migration behaviour, sex, reproductive status) and abiotic (salinity and temperature) factors might drive such differences by using a salinity change experiment.

In chapter 5, I explored how gut microbiome diversity and composition might differ among different populations, and what factors could influence these differences. I specifically focused on the sex and salinity as potential drivers. I also studied functional differences associated with microbial community composition between populations.

In chapter 6, I synthesized the results of all the previously described chapters and link all these studies together. In Chapter 6, I discuss and attempt to integrate the results of chapter 1 to 5 and suggest further analyses that could be undertaken in this system.

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Chapter 2: A study of intraspecific diversity in stickleback ecomorphs and their potential reproductive barriers in a tidal lagoon, Lough Furnace (Ireland)

Abstract

When populations occupy new environments, they can experience divergent selection, promoting the development of reproductive barriers, potentially resulting in decrease in gene flow between and among colonising and new populations. Since the last glacial maximum the three-spined stickleback, *Gasterosteus aculeatus*, has repeatedly colonised freshwater environments giving rise to populations that are reproductively isolated from their marine ancestors. However, in many places, secondary contact zones occur, where ancestral, emergent and hybrid populations co-exist resulting in a high level of polymorphism. In this study, I examined ecological and morphological differentiation among stickleback populations occurring in Lough Furnace a brackish lagoon in the Burrishoole river system located in the west of Ireland. A previous study in the lagoon suggested the presence of three distinct ecomorphs differentiated on the basis of phenotypic and genetic characteristics (i.e., low, partial and completely-plated), with little genetic evidence for hybridisation among ecomorphs. In contrast, I found sufficient morphological evidence only to support the presence of two ecomorphs in the system (a low and a completely-plated ecomorph). In Lough Furnace, the proportions of these two ecomorphs differed, with more low-plated, but overlapped both spatially and temporarily during the breeding season. I also found co-occurring mature females and males of both ecomorphs during the breeding season. Breeding experiments found that successful

interbreeding of ecomorphs was possible, at least between low-plated males and completely-plated females. However, no strong conclusions can be drawn because of some experimental design limits. In light of my findings, I discuss the role of the development of pre and post-zygotic reproductive barriers in ecological speciation, particularly in the context of explaining the nature of the populations inhabiting this secondary contact zone Lough Furnace. Also I discuss the apparent absence of hybrids despite potential opportunities for ecomorphs to interbreed.

Keywords: Sympatric speciation, ecological adaptation, phenotypic divergence, stickleback

Introduction

When colonising a new habitat, populations experience novel environmental pressures that can drive ecological speciation. Ecological speciation occurs when the evolution of reproductive isolation results from divergent selection on ecologically-linked traits in different ecological niches (Schluter, 2000a; Rundle & Nosil, 2005). Although much attention has been given to examples where ecological divergence drives sympatric speciation, ecological speciation can occur also in allopatry (fully geographically isolated populations) and parapatry (adjacent populations, with potential gene flow) (Ernst Mayr, 1963; Wu & Ting, 2004). As a consequence of this process, reproductive isolation develops and evolves between populations (Schluter, 2000a; Rundle & Nosil, 2005; Nosil, 2012; Faria *et al.*, 2014).

Identifying reproductive barriers that promote ecological speciation is challenging. The reproductive barriers can act either before reproduction (pre-zygotic barriers) e.g. habitat preference (Ahnesjö & Forsman, 2006; Bolnick *et al.*, 2009), allochrony (Hagen, 1967; Friesen *et al.*, 2007; Shimada *et al.*, 2011a), mate choice (Rundle *et al.*, 2000; McKinnon *et al.*, 2004) or after reproduction (post-zygotic barriers), e.g. selection against the hybrids (Coyne & Orr, 2004; Nosil, 2012; Kaufmann *et al.*, 2015; Matute & Cooper, 2021).

Secondary contact zones -- geographic regions where divergent groups overlap and have the opportunity to interbreed -- provide an excellent opportunity to study reproductive barriers. Investigating these zones can help us to assess the factors that regulate gene flow between groups and understand the continuum of speciation (Hendry *et al.*, 2009). Reproductive barriers usually occur across different ecological gradients, meaning that

one or more environmental factors change across space to form a continuum (Barton & Hewitt, 1985; Payseur, 2010).

The three-spined stickleback, has been commonly used to study ecological speciation as ancestral marine populations have repeatedly and independently colonised different freshwater environments (Wootton, 1984; Bell & Foster, 1994; McKinnon & Rundle, 2002; Hendry *et al.*, 2009). Marine-freshwater population pairs in three-spined stickleback are a classic case study for the concept of speciation continuum, showing any adaptive variation from within-population specialization to complete and irreversible reproductive barriers between species. Along this continuum, I can find ecomorphs (phenotypically differentiated groups adapted to local environments within the same gene pool), ecotypes (locally adapted and genetically-discrete groups representing different gene pools) or subspecies like the *Gasterosteus nipponicus* (Higuchi *et al.*, 2014). For example the partial or complete loss of lateral armour plates in freshwater environments (Wootton, 1976) has been observed repeatedly in different locations and several hypothesis have been proposed such as the response to the lack of calcium availability (Giles, 1983) or the different type of predators (Hagen & Gilbertson, 1973; McPhail *et al.*, 1973; Reimchen, 1992, 1995). This plate diversity has been reported since the early 1800s but, more recently the genomic basis of the changes have been explored (Peichel *et al.*, 2001; Colosimo, 2005).

There have been numerous studies on the nature of barriers in respect to reproductive isolation between specific stickleback pairs. For example in benthic-limnetic pairs in Misty Lake Canada, mate choice (Raeymaekers *et al.*, 2010; Räsänen & Hendry, 2014) and allochrony (Hanson *et al.*, 2016a) -- a change in breeding time which can reduce or eliminate gene flow between two populations -- were absent. However, they were present in other stream-lake systems, for example in studies undertaken in Northern Germany

(Eizaguirre *et al.*, 2011; Andreou *et al.*, 2017). Focussing on freshwater and marine anadromous stickleback pairs in a river and a tidal area in Scotland, Jones *et al.*, (2006) found very low levels of gene flow between the two ecomorphs, suggesting that post-zygotic barriers such as selection against hybrids must be important in this system.

Some pre-zygotic reproductive barriers, such as morphological incompatibilities and mate choice, can be observed under laboratory conditions but are more difficult to observe in the wild (Nosil *et al.*, 2002; Boughman *et al.*, 2005; Langerhans *et al.*, 2007; Lowry *et al.*, 2008; Castillo *et al.*, 2015). Nevertheless, these laboratory studies are unlikely to capture all the processes that might play a role in the wild. Some studies have therefore employed artificial but more realistic experimental set ups, such as mesocosms (i.e., experimental systems mimicking natural environments under controlled conditions) that aim to more closely reflect conditions that would be encountered in the wild (Schluter, 2000a; Via *et al.*, 2000; Hendry *et al.*, 2002; Schwartz *et al.*, 2010; Moser *et al.*, 2016).

A recent study of sticklebacks in Lough Furnace, a tidal lagoon in the Burrishoole system in the west of Ireland –based on both phenotypic and genetic criteria, found three different ecomorphs (Ravinet *et al.*, 2015). These three ecomorphs differed morphologically in respect of different anti-predator traits such as body shape, lateral plate number and pelvic and dorsal spines, but also for other phenotypic traits like gill raker length and number. These ecomorphs were described respectively as completely-plated, partially-plated and low-plated based on the methodologies reported by Munzing (1963) and Reimchen (2000) to discriminate plate ecomorphs. The completely-plated individuals had a higher number of lateral plates (>20) and a deeper body, whereas the partially-plated (7-20) and the low-plated (<6) had a lower number of plates with an elongated and shallow body. The completely-plated ecomorph was found to be strongly

genetically differentiated from the other two ecomorphs whereas the partially-plated and low-plated were not. This held for both neutral and QTL microsatellites markers. The QTLs were linked to the EDA (ectodysplasin) gene region has been shown to be strongly associated with variation in armour plate phenotypes (Colosimo *et al.*, 2004, 2005). Population genetic analyses showed that there was very little genetic evidence of hybridisation among the three ecomorphs identified in Lough Furnace, but the mechanism or mechanisms preventing gene flow in this system were not known. Ravinet et al. (2015), did suggest, however, that assortative mating between lateral plate phenotypes was the most plausible explanation, but that temporal and spatial isolation in spawning period was also a possibility.

Ravinet et al. (2015) postulated that the completely-plated sticklebacks were likely to be 'anadromous': living in the ocean and entering Lough Furnace specifically to spawn. Further, the low-plated phenotype was postulated to have originated from the freshwater portion of the Burrishoole system, most likely displaced without possibility of return due to physiography of the glacial moraine that separates the freshwater Lough Feeagh from the tidal lagoon Lough Furnace. Finally, the partially-plated ecomorph was possibly a resident of Lough Furnace adapted to brackish conditions and distinguished by a larger body size and to have an intermediate number of lateral plates. The sampling by Ravinet *et al.* (2015) was confined to the breeding season from March to June and hence was inconclusive whether the completely plated ecomorph was migrating in and out of the lake exclusively for the breeding season. As migratory behaviour can be very complex in this species (anadromy, partial anadromy, estuary resident) (Kitano *et al.*, 2012), it would be important to sample more extensively, both temporally and spatially within and outside of the breeding season, including identifying and sampling truly marine and geographically proximate archetypical sticklebacks.

Here, I re-examine the biology of the stickleback in Lough Furnace by undertaking a much more extensive study of the lake by (1) collecting and analysing new samples across different years to characterise the level of phenotypic diversity (meristic differences in plate composition and morphological difference in body shape between individuals) in the lagoon's sticklebacks and comparing these to baseline ecomorphs sampled from the freshwater population in Lough Feeagh and marine ecomorphs sampled in shoreline rock pools located along the neighbouring coastline; (2) comparing the temporal and spatial distribution of the different ecomorphs by way of a standardized monthly monitoring sampling programme deployed over two years; (3) determining the numbers and proportions of reproductively mature female and male individuals in respect of plate morphology sampled during the breeding season; (4) carry out a series of breeding experiments under environmentally enriched artificial semi-natural conditions to ascertain whether there exist inherent pre-zygotic barriers (e.g. assortative mating) or post-zygotic barriers (e.g. genetic incompatibilities) to successful reproduction between ecomorphs.

Methods

1. Study area

The Burrishoole catchment (N53°55′22″, W9°34′20″), located in the west of Ireland in Co. Mayo, is composed of different rivers and lakes connected to the Atlantic Ocean via Clew Bay. The two main lakes are Lough Feeagh (410 ha) and Lough Furnace (141 ha) (Poole & de Eyto, 2006; Cassina, 2012). Lough Feeagh is an oligotrophic freshwater lake with a maximum depth of 46 m (de Eyto *et al.*, 2016). Feeagh is connected to Furnace by two channels transferring freshwater water across a substantial relic glacial moraine:

the natural Salmon Leap and the artificial Mill Race, probably constructed in the 16th century. Lough Furnace is a stratified and brackish coastal lagoon with a maximum depth of 20 m (Cassina, 2012; Cassina *et al.*, 2013). Lough Furnace presents very diverse and interconnected hydrological and chemical characteristics: a stable stratified salinity layer between the surface and deeper water maintained by a strong and shallow pycnocline (~2.4 m) with high concentrations of dissolved oxygen in the surface, but anoxic conditions below ~6-7 m depth; both temperature and oxygen can change in a single day with a mixing of deeper water layers with the surface and strong winds causing important upwelling events in the lake (Kelly *et al.*, 2016, 2018). Lough Furnace displays a strong salinity gradient with low concentrations in the north part of the lake (from 2 to 4 PSU at the surface) to brackish concentrations in the channel that connects to Clew Bay (10 to 15 PSU). In comparison, Lough Feeagh is a purely freshwater lake (0 PSU) that stratifies in summer and is influenced by rainfall and river floods. The diurnal and seasonal changes in Lough Feeagh are not as drastic as those observed in Lough Furnace. Clew Bay, the estuary into which Lough Furnace exits connecting it to the Atlantic Ocean, is an area of high habitat diversity and has been designated a Special Area of Conservation (SAC Site Code 001482, under the EU Habitats Directive; National Parks and Wildlife Service 2011). A marine site, consisting of rock pools (20 to 35 PSU), was located along the Muingdoran seashore in Tullaghan Bay, near the town of Belmullet Co. Mayo, approximately 50 km from where Lough Furnace enters the sea.

2. Sample collection

In 2018 and 2019, a monthly sampling campaign for sticklebacks was carried out from March to August in the Burrishoole system (Figure 2.1b), completed with two other samplings (one in autumn and one in winter for both years). The sampling sites were

distributed across the catchment: Srahrevagh (Rough) River, Lough Feeagh and Lough Furnace. The Muingdoran marine/rock pool site was also fished from July to October 2019 and in May 2020 (Figure 2.1a). Unbaited minnow traps (silver galvanized Gee-type model #1279 Frabill, Jackson, Wisconsin, USA) were set and left for 48 hours. When sample sizes allowed, ten fish of each of two putative low and completely-plated ecomorph and each sex were chosen per site and taken back to the lab. Selection of each morph and sex was done by sight on location at the sampling site. Intermediate morphs were also collected. Surplus captured fish were counted and released (S2.2). Additional samples from Muingdoran (n=34) and the Burrishoole system (n=113) were collected in May 2020 and were added to the main dataset. Fish were euthanized with a concentration of 1.0 g/L of MS-222 solution (Tricaine methanesulfonate, FVG Ireland). Standard length (from the snout to the fork), total length, weight and body height were measured and lateral plate number counted. All measures were recorded to the nearest mm and g. The lateral plate number was counted on both sides using a binocular microscope and the average was calculated for each fish. Extra samples from 2009 and 2010 collected by Ravinet *et al.* (2015), were used for this study.

This research was approved by the animal ethics and welfare bodies of University College Cork and the Marine Institute, and conducted under Health Products Regulatory Authority (HPRA) license number AE19130-P096.

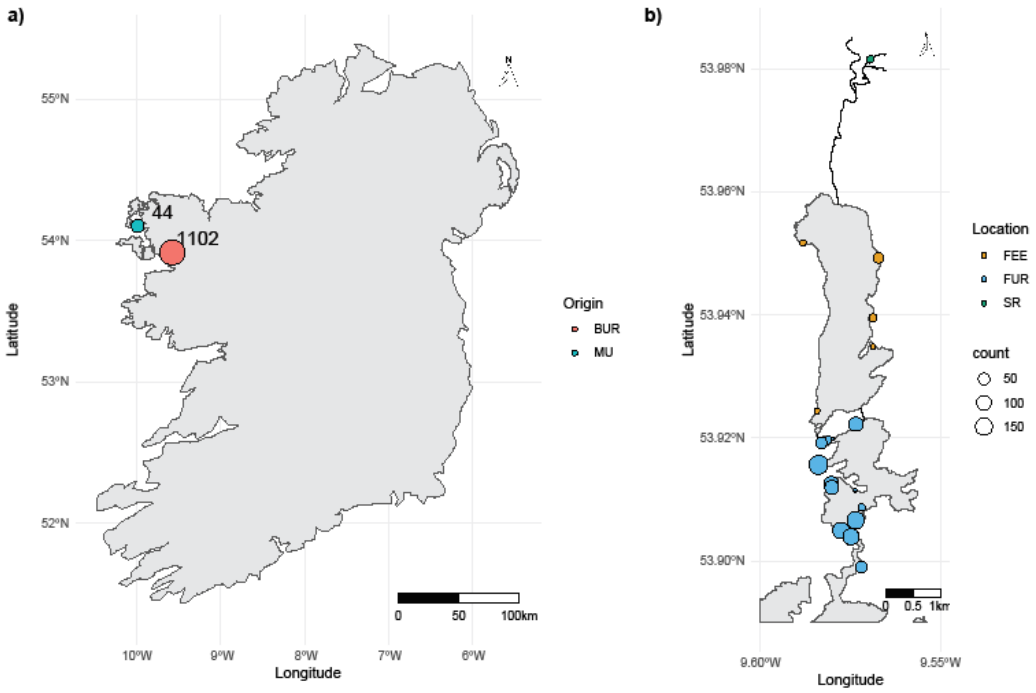

Figure 2.1. a) Map of Ireland showing the two main locations on the west coast of Ireland: Muingdoran (blue) and Burrishoole (red) with the number of fish caught per location; b) The Burrishoole system with the three main locations: Lough Feeagh (orange), Lough Furnace (blue) and Srahrevagh River (green). The circle size is proportional to the number of fish caught at a given site.

3. Ecomorph identification and morphology

a) Phenotypic clustering methods

To assign an ecomorph to each individual in the dataset, a clustering method was used with Mclust package in R v 3.4.2 (L. Scrucca, M. Fop, 2016). Where data on total length or count for lateral plate number was missing individuals were excluded. Only sexually mature individuals were used for ecomorph assignment purposes. Fish sampled in Burrishoole with a total length less than 30 mm were excluded, assuming the smaller fish to be juveniles and their lateral plates not completely developed (Bell, 1981; Banbura, 1989). For fish sampled at the Muingdoran site, data exploration indicated that fish less than 50mm in length could be considered to be juveniles (gonads not developed). I also

removed six individuals from Burrishoole that were defined as outliers based on data exploration and distribution (S2.3). The different functions of the MClust package combine models based on hierarchical clustering, expectation-maximization (EM) algorithm and on the Bayesian Index Criterion (BIC) for clustering. The first BIC estimation was made based on two variables only: total length and the number of lateral plates. A second BIC estimation was done by adding the PC1 to the other variables in the model. PC1 is the first component of the morphological analysis based on the 20 landmarks (see 2.3.b). The other variables (height and weight) were completely correlated with the previous variables, total length and number of lateral plate (Pearson test, cor = 0.56, $P < 0.01$), and were therefore not used in this clustering analysis. The two models were undertaken separately.

b) Morphological analysis

For each sampling event (2018, 2019 and 2020), a representative subset of sticklebacks (with a length and plate range variation) were selected randomly and photographed on their right side using a CANON 1300DC camera with a macro lens. The photographs were taken at a standardised distance and a ruler was placed for scaling. After excluding females with swollen abdomen (n=125) and any poorly focused pictures (n=151), 629 images were kept for morphometric analysis. Twenty digitized landmarks were placed (Figure 2.2) using the software tpsDig2 (Rohlf, 2008). The positions of landmarks were analysed using the R package geomorph (Adams & Otárola-Castillo, 2013). First, I performed a Generalised Procrustes Alignment (GPA) to remove differences in fish size, orientation, and position. I then ran a principal component analysis (PCA) to understand the major axis of variation, and used the deformation grid produced in geomorph to

represent the shape differences between the mean shape reference and the maximum and minimum values of PC1.

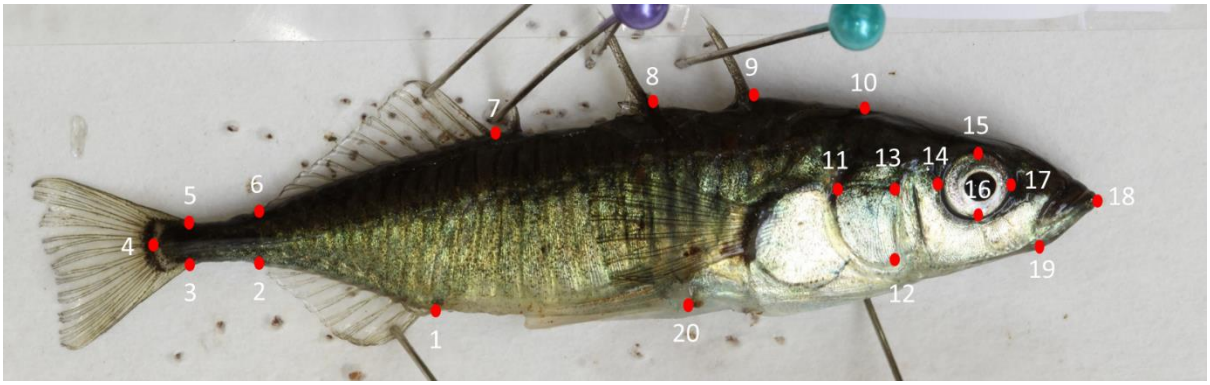

Figure 2.2. Position of the 20 landmarks, which refer to : (1) origin of ventral fin near the anal spine, (2) end of the ventral fin near the tail, (3) insertion of ventral caudal fin ray, (4) posterior tail, (5) insertion of caudal fin ray, (6) insertion of dorsal fin near the tail, (7) end of dorsal fin near the third spine, (8) origin of second spine, (9) origin of first spine, (10) section between the frontal and the supra-occipital bone, (11-13) operculum, (14-17) eye, (18) snout, (19) outline of ventral jaw (20) anterior of pelvic spine.

c) Synchrony in female and male maturity

During the breeding season from April to July in 2018 and 2019 and in May for 2020, all females caught in Lough Furnace (n=533) were euthanized and subsequently dissected to evaluate their state of maturity. The presence of a swollen body cavity and presence of eggs in the ovaries were recorded to establish maturation status.

During the same month in 2019 and in May 2020, males caught in Lough Furnace (n=268) were also euthanized and subsequently dissected to evaluate their reproductive status. The presence of nuptial colours (orange/reddish throat and blue eyes) and the observation of developed testes were recorded to establish their maturity status.

d) Mesocosm breeding study

Tanks used to establish the breeding mesocosms were 3.36 m in circumference and filled to a depth of 30 cm with freshwater supplied from Lough Feeagh (S2.1). Tanks were cleaned and sanitised before use. Each tank environment was enriched with: ten squares (22 cm x 22 cm) of synthetic grass (Wondergrass©), taped at the bottom and placed at equal distance from each other (S2.4 and S2.5), imitation of a plant (made with a plastic bag filled with sand) was placed on a piece of grass for each square. To delimit different potential nest areas, each grass square was surrounded by two pieces of black plastic pipe (each containing ca. 2 stones as ballast). Under experimental conditions, the best material for nest construction by males is a mixture of sand and gravel with cotton or nylon threads between 5 to 10 cm long (Barber *et al.*, 2001; Little *et al.*, 2008). The same amount of nest material was provided on each grass square: one petri dish containing 100 g of aquarium sand and 80 g of 6 cm long green nylon threads (Stewart *et al.*, 2017). All enrichment and nesting material was disinfected before use. Once the fish were released, a net (with a mesh size of 4 x 4 cm) was put on top of each tank to prevent access to predators (like birds and mustelids). At the start of the experiment, males were released first in the tank to adjust to their new environment and set up their territories. Meanwhile, the females were kept in buckets for an extra 24 hours before being introduced into the tank. To reach the required number of fish in the tank (ten males and ten females per tank), fish were continuously collected and introduced in the tanks after acclimation.

For the mesocosm breeding study, I collected individuals typical of the two ecomorphs identified in Section 2.3.a, following the methods detailed in Section 2 (minnow traps were baited with cheddar cheese) at three neighbouring sites where both ecomorphs had been found to coexist in 2018. The sampling sites chosen in Lough Furnace where those known to have a relatively stable and low salinity level (S2.6), facilitating and reducing

the duration of the acclimation prior to the breeding studies. Traps were checked every 24 hours to remove predators (eels and crabs) from entering the traps. Fish were collected and transported in an aerated bucket (at a density of 6 fish per 5 litres). Fish were anaesthetised with a concentration of 50-75 mg/L MS-222 solution in the lab (Tricaine methanesulfonate, FVG Ireland) to sort the fish into ecomorph (low-plated and completely-plated, based on the number of plate) and sex. Only sexually mature fish were kept for the experiment, i.e. males with breeding colours (orange-reddish throat and blue eyes) and gravid females. Fish were held for 24 h in previously disinfected buckets containing half Lough Furnace water (from their site of origin) and half of Feeagh (i.e. freshwater) to acclimate. The fish were then transferred in buckets with water (25% Furnace and 75% Feeagh) in order to reach a pure freshwater equivalent to that acquired from Lough Feeagh. Males were kept in individual buckets to reduce aggressive behaviours, whereas three or four females were kept in the same bucket. Twenty-four hours later, fish were released into four different mesocosms using the following experimental design. To evaluate the reproductive potential of all ecomorphs, males from each ecomorph were introduced into tanks with either females of a similar ecomorph or the alternative ecomorph. This resulted in four different potential breeding combinations: 1) completely-plated females x completely-plated males; 2) low-plated females x completely-plated males; 3) completely-plated females x low-plated males; 4) low-plated females x low-plated males (S2.7). Captured individuals were placed progressively into the tanks to reach (when possible) a number of ten males and ten females in each tank. Ideally, replicates would have been undertaken for each breeding group combination to account for potential tank effects, but unfortunately due to the limited number of completely-plated males caught in the field, only one tank per cross type was possible. Further details about fish care and welfare can be found in appendices (S2.1). The

breeding experiments took place from May 2019 until the end of July 2019. Individuals presenting abnormal behaviour (i.e. swimming close to the surface, unresponsive when probed, no feeding interest) were removed from the tank and euthanized using an overdose of 1.0 g/L of MS-222 solution in the lab (Tricaine methanesulfonate, FVG Ireland). High levels of mortality were observed following spawning similar to what would be expected to happen in the wild (Wootton 1984). To keep the experimental population density constant and compensate for mortalities, freshly caught sexually mature individuals were introduced in the tanks on a bi-weekly basis. Morphological measurements were taken on mortalities, surviving fish and offspring at the end of the experiment, following the same methods as above. During the experiment, the presence and numbers of nests constructed was recorded every second day. At the end of the experiment, I also carefully removed all the material and collected juveniles that were hiding in the pipes.

4. Statistical analyses

To test the influence of body length, sex and ecomorph on shape, I used a MANCOVA on the three first component of the PCA analysis. Phenotypic traits (i.e., total length, number of lateral plates), were analysed separately using a linear mixed models with sex (F vs M) and ecomorph (completely-plated vs low-plated) as fixed effects and location (Muingdoran, Lough Furnace, Lough Feeagh and Srahrevagh River) as a random effect to avoid a group effect, using the lme4 package in R. As per Ravinet al. (2015), I created a variable containing the plate morph (completely-plated and low-plated) and the origin of the fish (Srahrevagh River, Lough Furnace, Lough Feeagh and Muingdoran), yielding five groups (completely-plated Muingdoran, completely-plated Lough Furnace, low-plated Lough Furnace, low-plated Feeagh and low-plated Srahrevagh River).

To test for temporal variation in the frequencies of completely-plated and low-plated ecomorphs in Lough Furnace, I used a Pearson's Chi-square assessing the homogeneity of the completely-plated and low-plated proportions across months for all captured individuals (mature or immature). To test the synchrony between ecomorphs (i.e. females and males from both ecomorphs are sexually mature at the same time), I used a Pearson's Chi-square to test the homogeneity of completely-plated and low-plated gravid females and coloured males proportions separately, across months.

To ascertain if there were differences in the frequency of nest building and numbers of juveniles produced, a comparison was made using Pearson's Chi-square across each experiment.

Results

1. Ecomorph identification and phenotypic variability

I collected a total of 1,146 sticklebacks over three years (2018, 2019 and 2020) at four locations (Figure 2.1a). At the sea site at Muingdoran, 44 fish were caught in fully saline rock pools along the shoreline in 2019 and 2020. In the Burrishoole, 9 fish were collected in the Srahrevagh River, 83 in Lough Feeagh and 1,010 in Lough Furnace (Figure 2.1b).

The first two axes of the principal components analysis explained 40.6% of the total variation in body shape. The first principal component explained 24.1% (Eigenvalue= 0.0005) and differentiated fish presenting a large and deep body from fish with a shallower and thinner body (Figure 2.3). The second principal component explained 16.5% (Eigenvalue=0.0003) and linked to the depth of the body cavity.

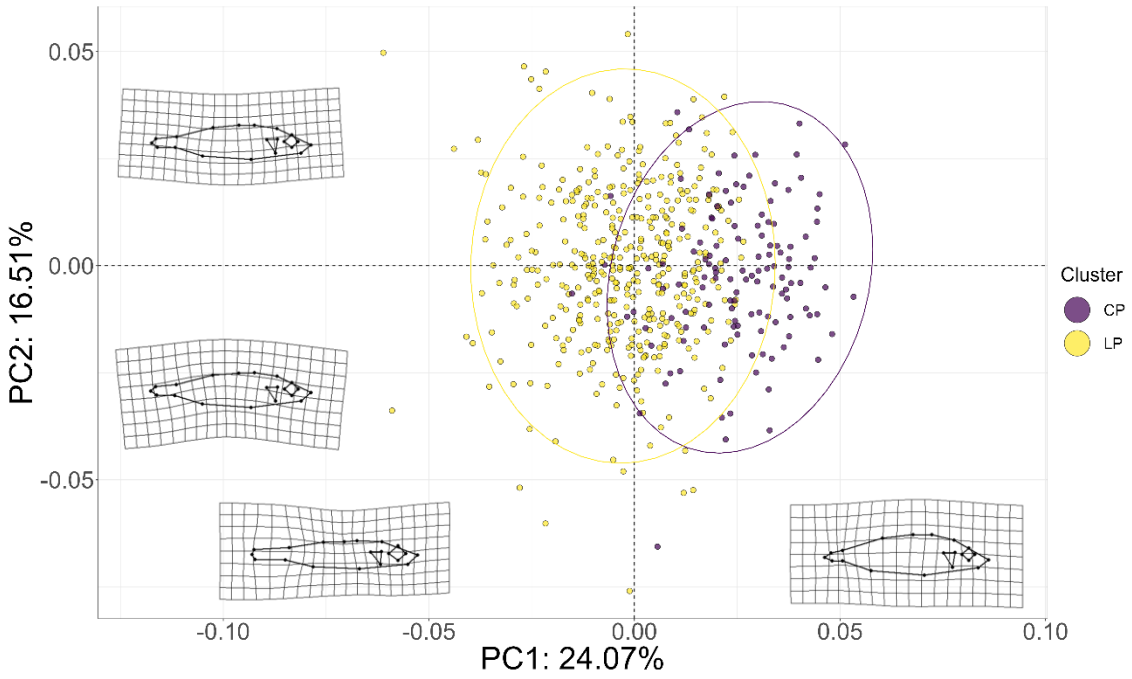

Figure 2.3. Geometric morphological divergence among the two clusters identified using the best MClust model (completely-plated and low-plated) with the deformation grid representing the two extreme shape differences along the PC axis. Each point represents an individual. Coloration represents the ecomorph each individual was assigning to: completely-plated (purple) and low-plated (yellow).

The first model included individuals from Muingdoran and the Burrishoole (n=974) and was based on two variables: total length and lateral plate number. The best model was VVE (ellipsoidal, equal orientation) with two clusters (df=9, BIC=-10665.76, S2.8). Individuals grouped in the first cluster had an average total length of 44.9 mm and an average plate number of 5.3 plates per side. Individuals in the second cluster had an average total length of 65.4mm and an average lateral plate number of 28.8 plates per side (S2.9). The second model included individuals from Muingdoran and the Burrishoole (n=529) and was based on three variables: total length and lateral plate number and morphological shape information (PC1). The best model was EVE (ellipsoidal, equal volume and orientation) with two clusters (df=15, BIC=-2792.9, S2.10). Individuals grouped in the first cluster had an average total length of 44 mm and an average lateral plate number of 5.6 plates per side (low-plated), whereas the second

group had an average total length of 64.8 mm and an average lateral plate number of 28.7 plates per side.

Fish were thus grouped into two ecomorphs: a completely-plated cluster with a high average total length and a high average of lateral plate per side, and a low-plated cluster with a lower average total length and a lower average lateral plate per side (S9). The next two models which included only adult fish from Lough Furnace, with (n=459) and without (n= 825) morphological shape information (S2.11 and S2.12) provided similar results to analyses with all the individuals.

Body shape was influenced by total length ($F_{1,397}$=18.1, P < 0.001), group (morph-location combination) ($F_{4,397}$=23.6, P < 0.001), and sex ($F_{1,397}$=30.2, P < 0.001). I observed that the total length was influenced by ecomorph (R^2=0.62, $F_{1,882}$=992.3, P < 0.001, CP >LP) and sex ($F_{1,976}$ =117.4, P < 0.001, F>M). The number of lateral plates was influenced by the total length of the fish ($F_{1,976}$ =11.4, P < 0.001) and the ecomorph ($F_{1,976}$ =4864.5, P < 0.001, CP>LP) but sex did not seem to influence the number of lateral plates ($F_{1,976}$ =2.2, P = 0.14).

2. Spatial and temporal distribution of ecomorphs across the Burrishoole system

The two ecomorphs (i.e., completely-plated and low-plated) differed in their spatial distribution over the sampling area. At Muingdoran, only completely-plated individuals were captured. In Lough Feeagh and in the Srahrevagh River, only low-plated individuals were captured. Lough Furnace was the only location where both ecomorphs were present together (Figure 2.4a). The distribution of ecomorphs was quite similar across years (Figure 2.4b). In 2020, I sampled opportunistically in May only and therefore, I did not collect data across months.

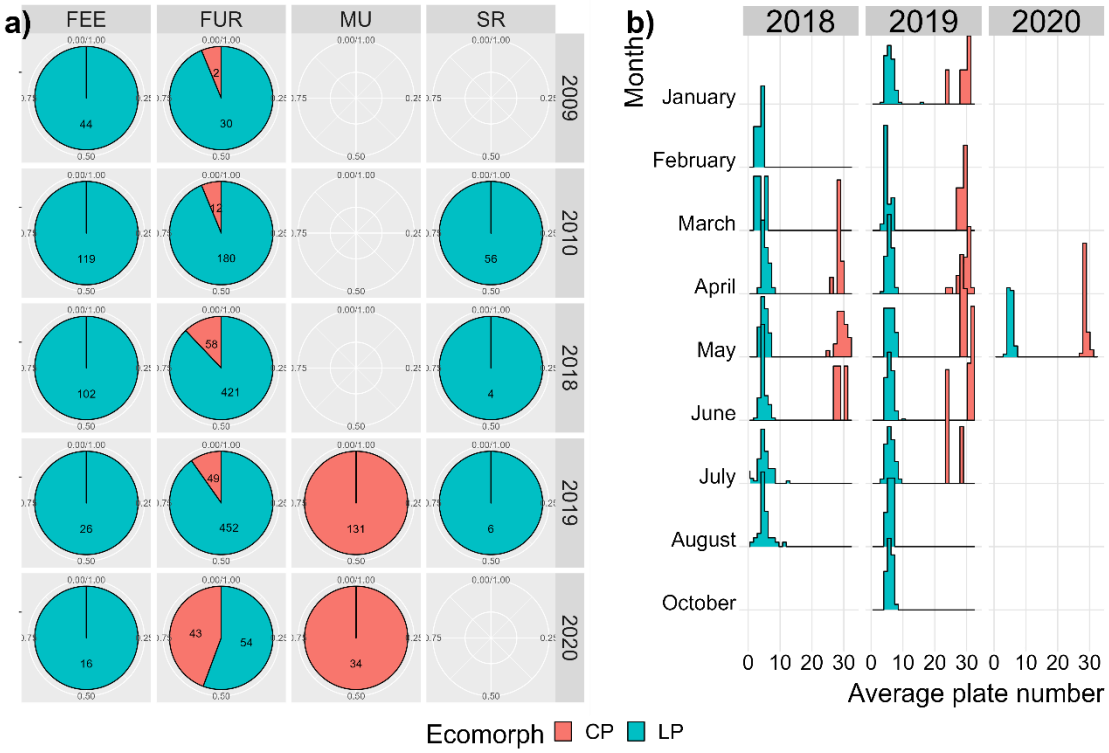

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Figure 2.4. a) Distribution of ecomorphs (red = completely-plated; blue = low-plated) across locations and years; b) Lateral plate number distribution across months in 2018, 2019 and 2020 in Lough Furnace.

385 When focusing only on Lough Furnace, where I could observe both low-plated and completely-plated, I established that completely-plated individuals were not always present in the lake. In contrast, low-plated individuals were observed in every month of the year, while completely-plated individuals were only reported from March to July (Figure 2.5b). There was an exception in January 2019 as a few completely-plated were 390 caught (n=5). I found that both completely-plated and low-plated were present in Lough Furnace at all sampling sites during the breeding season (Figure 2.5a). The same phenomenon was observed for both females and males for the two ecomorphs. In 2018 and 2019, I found that the proportions of completely-plated and low-plated were dissimilar across months (χ_{28}=134.4, P <0.001). The proportion of completely-plated fish 395 was lower than low-plated fish at all times, including during the peak of the breeding

season in May with a maximum of 38% of completely-plated across all the sites (Figure 2.5b).

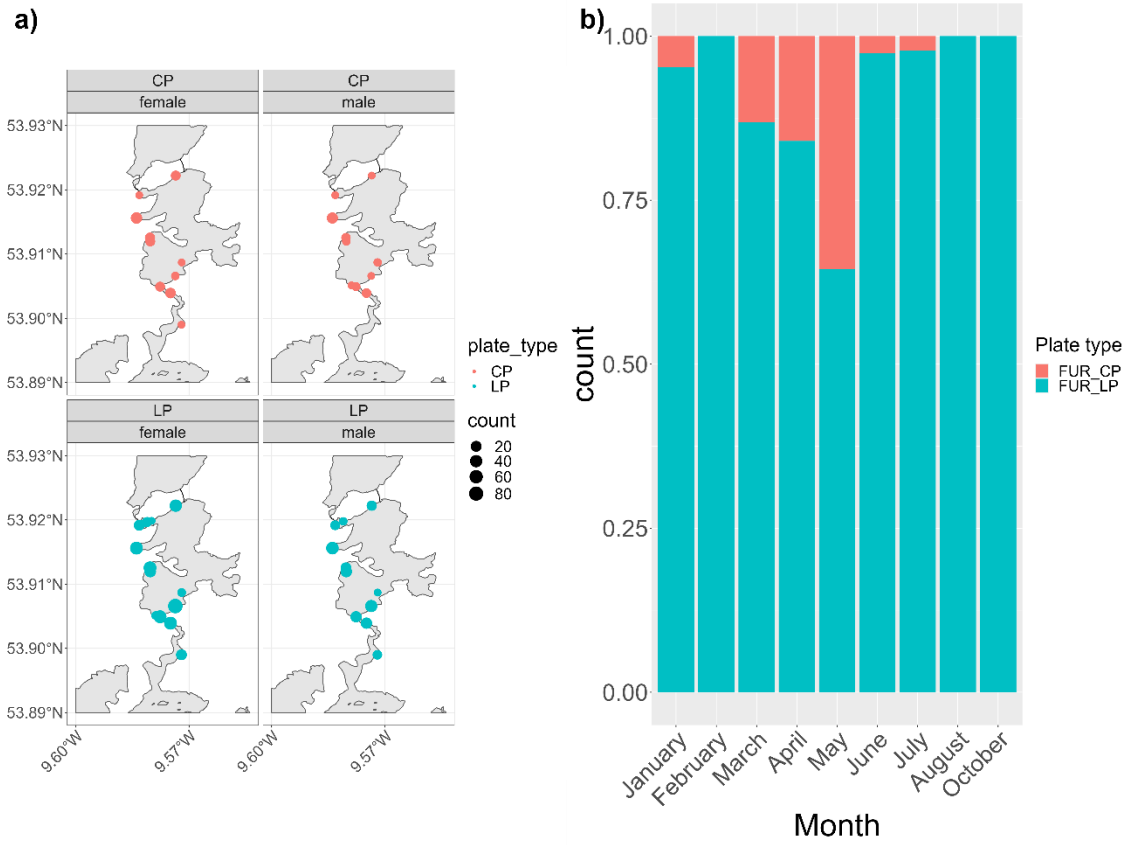

Figure 2.5. a) Ecomorph and sex distribution across different sampling sites in Lough Furnace. Circle size represents the number of fish caught; b) Ecomorph proportions per month for 2018 and 2019 only.

3. Synchrony in female and male maturity

In respect of the timing of female and male spawning readiness, I considered only the four-month period, April to July (the peak of the breeding season) when the completely-plated females are also abundant in the system (n=108). The low-plated females being constantly present in the system (n=425). This period is also when I observed the gravid females to be most abundant, even though four of them (four low-plated) were captured in August (end of the reproduction season). I found a total of 76 gravid completely-plated

females relative to 270 gravid low-plated females. I compared the homogeneity of the proportion of gravid females of each ecomorph across months (Figure 2.6a). The proportion of gravid females differed between low-plated and completely-plated groups across these months (χ_3=1185, P <0.001). Nevertheless, the proportion of gravid females in each group was high. For example, in April, the majority of caught females were gravid in both ecomorphs (100% of completely-plated females were gravid and 77% of low-plated females were gravid). For males, I also considered the same four months of the breeding season when both completely-plated (n=58) and low-plated males (n=210) were captured in Lough Furnace. I compared the homogeneity of coloured males (completely-plated and low-plated) proportions across months. The proportion of coloured low-plated and completely-plated males were both high, especially in June and July, but were found to be different (χ_3=7909, P <0.001). In June, both completely- and low-plated coloured males were high (100% of completely-plated males were coloured and 95% of low-plated males) (Figure 2.6b).

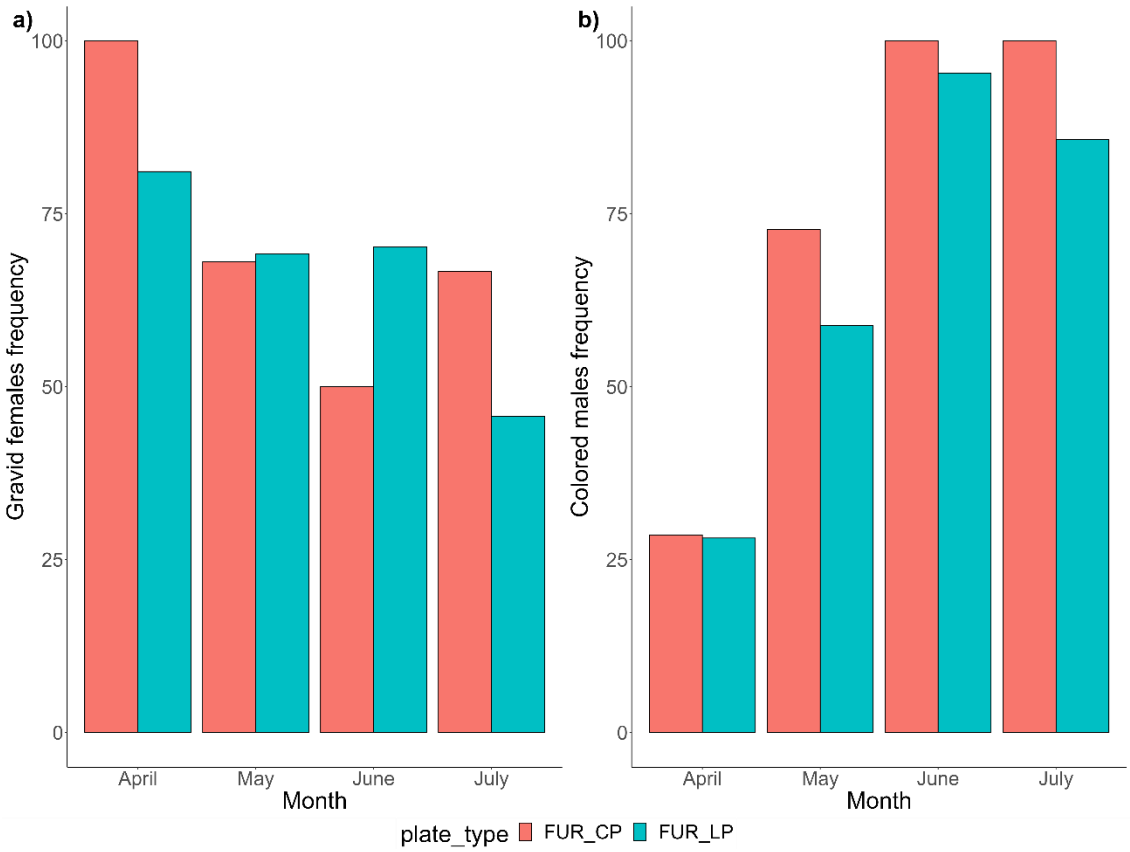

Figure 2.6. a) The frequency of gravid females from April to July for the completely-plated (red) and low-plated (blue) ecomorphs in Lough Furnace; b) The frequency of coloured males from April to July for completely-plated (red) and low-plated (blue) ecomorphs in Lough Furnace.

4. Breeding studies

All individuals were kept in the same conditions but their likelihood of surviving until the end of the experiment differed (χ_3=10.9, P=0.01). Both completely-plated females and males died during the experiment (final cumulative mortality = 100%) whereas some low-plated females and males survived until the end (female final cumulative mortality = 66% and male final cumulative mortality = 74%) (S2.13). I found a substantial difference in nest building between the groups (χ_3=8.5, P=0.003). Completely-plated males built three nests with low-plated females and none when placed together with completely-plated females. Low-plated males built eight nests with completely-plated

females and five when placed with low-plated females. Altogether, low-plated males built 13 nests, which was 5.7 times more than completely-plated males (three nests). I also observed differences in juvenile production between the groups (χ_3=52.6, P<0.001). Completely-plated males produced no juveniles whereas low-plated males produced 29 juveniles when placed with completely-plated females and 21 when placed with low-plated females.

Proportionally, relative to the number of males introduced into the tank per ecomorph, 29.3% of low-plated males produced a nest, whereas 12.5% of completely-plated males produced a nest.

Discussion

This new study of the stickleback complex in the Burrishoole system confirms the presence of more than one ecomorph associated with lateral plate morphology in Lough Furnace – a brackish lagoon connected to the Atlantic Ocean. Using morphological data, I suggest two distinct ecomorphs – a completely-plated marine type fish and a low-plated freshwater phenotype. I found no supporting evidence, for a distinct partially-plated resident ecomorph as proposed by Ravinet *et al.* (2015) at least based on morphological attributes. The completely-plated ecomorph was similar in plate composition and body morphology to stickleback found spawning in fully-marine water rock pools located along the neighbouring shoreline approximately 50 kilometres from where Lough Furnace enters the sea. The low-plated ecomorph observed in Lough Furnace was comparable to that found in Lough Feeagh, a freshwater lake in the Burrishoole system directly upstream of Lough Furnace, and in the Srahrevagh River, which flows into Lough Feeagh. Both ecomorphs overlapped in Lough Furnace in time and space with reproductively mature individuals of each sex and of both ecomorphs present during the

breeding season. Moreover, when Furnace low-plated and completely-plated were placed in semi-natural mesocosms as part of a breeding study, low-plated males produced nests and juveniles with both completely-plated and low-plated females, whereas high-plated males only produced nests with low-plated females but produced no offspring. The results of the study are discussed below, in general, with regard to development of pre- and post-zygotic reproductive barriers during secondary contact, their role in ecological sympatric speciation, and in particular the apparent absence of hybrids inhabiting the recently characterised Lough Furnace stickleback contact zone.

Ecomorph diversity in a complex environment

Ravinet *et al.* (2015) proposed that there were three different stickleback ecomorphs coexisting in Lough Furnace in 2009 and 2010, i.e. low-plated, completely-plated and partially-plated. These three ecomorphs were phenotypically differentiated by lateral plate number, body shape and gill raker morphology. They found that while gill rakers did not differ in number among ecomorphs, there were differences in gill raker length standardised for size such that the low plated fish had the shortest gill rakers relative to their size. These results were similar to the findings of other studies based on gill raker comparisons (Bentzen et al., 1984; Schluter, 1993; McPhail, 1994; McKinnon & Rundle, 2002) and suggest that ecomorph variation might result from specializations on different food items either following colonisation of the freshwater environment after the last glacial retreat or during secondary contact. These differences may have evolved in response to differences in prey type availability in the different environments and could be the reason why low-plated individuals in Lough Furnace are bigger in size than low-plated individuals in Lough Feeagh and in the Srahrevagh River.

Based on the extensive and substantially increased sampling undertaken during the breeding season in 2018 and in 2019, I could differentiate here using clustering methods based on body morphology measurements two ecomorphs, (completely-plated and low-plated, but could not find evidence to support the partially-plated ecomorph suggested by Ravinet *et al.* (2015) based the same metrics. There are a number of possibilities that might explain the presence of a partially plated ecomorph in the earlier study.

First, the young of a completely plated morphs may be misidentified as specimens of low or partial morphs (Banbura, 1989). It could be accordingly that the partially-plated individuals identified by Ravinet *et al.* (2015) were completely-plated ecomorph juveniles that had not yet developed fully their final complement of lateral plates. Lateral plates are generally considered to be fully developed when the fish body length is greater than 30 mm (Bell, 1981; Banbura, 1989), though this is likely to be population specific and predominantly observed in studies of freshwater low plated forms. However, in this study I observed that most completely-plated individuals sampled in Muingdoran and Lough Furnace, developmental status being decided on the basis of whether individuals were sexually mature or not, could not be considered to be fully developed adults until their body length exceeded 50 mm. Juveniles (immature individuals) in Muingdoran were found to range in size anywhere from 15 mm to 45 mm and to present a variety of plate phenotypes. Most of the partially-plated ecomorph individuals described in Ravinet *et al.* (2015) had a body length less than 50 mm (the average body length of the partially-plated ecomorph was 39.3 mm). However, the genetic data presented by Ravinet *et al.* (2015) does not align with this interpretation by assigning low and partially plated fish sampled in Lough Furnace to the same genetic cluster.

A second possibility could be that the partially-plated phenotype had disappeared from Lough Furnace in the ten years separating this study and the earlier study (2009-2010 vs

2018-2020). Phenotypic change and the disappearance of particular morphs over a short time period has been observed previously (Kitano *et al.,* 2008). They suggested an evolutionary reversal in a period of 40 years between 1968 and 2005, where the proportion of the low-plated ecomorph relative to the completely-plated ecomorph decreased from 70% to 16%, probably caused by an improvement in water clarity facilitating an increase in subsequent predation on the low-plated individuals. Other studies have highlighted large changes on even shorter time scale e.g. from 1990 to 2001, e.g. Bell *et al.*, (2004) observed a strong increase of the low-plated ecomorph from 0% to 75% from a starting (almost monomorphic) population that consisted of 96% completely-plated and 4% partially-plated ecomorphs. The completely-plated ecomorph fell to 11% of the population in 2001, with the appearance of other rare intermediate type ecomorphs. In an experimental pond, Kristjánsson (2005) showed that after one year in a freshwater environment, marine stickleback offspring had lower lateral plate number than their parents as well as differing morphologically. This rapid morphological change could be explained either by phenotypic plasticity or the negative selection imposed by the freshwater environment on marine completely-plated stickleback. Moreover, as observed in our breeding experiments freshwater may not be optimal for the completely-plated stickleback (discussed below). All these examples indicate that changes in ecomorph distribution can be very responsive to different environmental pressures and environmental change.

Partial anadromy suspected among the completely-plated Lough Furnace stickleback

I found no differences in plate morphology between the completely-plated ecomorph found in Lough Furnace and those individuals sampled in the Muingdoran rock pools. Interestingly, with the exception of a few individuals captured in Lough Furnace in

January 2019, both were observed in their respective sampled environments only during the breeding season: around March-April to late August. Those few individuals captured during the winter in Lough Furnace could potentially be resident (e.g. hiding in deeper water and hence avoiding capture) during winter before coming near to the shore in the spring to breed. The samples collected from the fully saline rock pools at Muingdoran, an ephemeral ecosystem subject to the daily fluctuations of tidal cycles, without any connection to a freshwater system, suggests a different possibility. Sticklebacks were observed in the rock pools exclusively during the breeding season and not during winter months (Ross Finlay, personal communication). They must, therefore, come into this particular habitat from the sea to breed and leave subsequently. Completely-plated sticklebacks from Lough Furnace may be exhibiting a similar seasonal behaviour associated with the requirement to reproduce: entering into Lough Furnace to breed and returning to the sea afterwards. In Lough Furnace, it may be that this seasonal behaviour in completely-plated individuals is typical, but given that I observed (even a few) individuals during winter, it leaves the question open to the exclusivity of this behaviour. It may be that partial anadromy occurs, where some individuals are anadromous and migrate whereas the others are estuary-resident and do not migrate, which has been observed in other systems (Kitano *et al.*, 2012). A recent study by Arai *et al.*, (2020) used the otoliths Sr:Ca ratios to identify life-history migration and showed that morphological observation does not reflect the actual migration behaviour. Indeed, they observed that 90% of sticklebacks identified as anadromous in both stickleback species (*G. aculeatus* and *G. nipponicus*) based on their morphological traits, are actually estuary residents and never migrate to freshwater habitats. Only the other 10% are actual anadromous sticklebacks. Further investigations of the completely-plated ecomorph migration pattern in both Lough Furnace and the Muingdoran would be worthwhile to resolve the

exclusiveness or otherwise of this behaviour. Additionally, investigating variation in ecological bio-markers such as stable isotopes (e.g. Sr:Ca ratio), biochronological depositions (e.g. otoliths) or signature microbiome community could provide evidence of differences in migratory behaviour (Arai *et al.*, 2003).

Potential second contact zone in the Burrishoole system

In the Pleistocene, most of Ireland was covered in ice (McCabe *et al.*, 2007; Ballantyne *et al.*, 2008) with sticklebacks believed to recolonise rapidly into newly released freshwater environments following the retreat of the ice (Ravinet *et al.*, 2014). Ravinet *et al.*, (2015) hypothesised that sticklebacks would have quickly recolonised Lough Feeagh and Lough Furnace, but as sea levels were fluctuating widely during this period (Cassina *et al.*, 2013), there might well have been a substantial period of time when freshwater stickleback were completely isolated from the sea and hence from their marine congeners. More recently, sea levels have increased (Cassina *et al.*, 2013), restoring the connection between Lough Furnace and the ocean, facilitating marine sticklebacks to migrate into Lough Furnace and co-occur with residents, resulting in a secondary contact zone. This scenario has been observed in other systems where marine anadromous and freshwater resident stickleback pairs overlap in the same place (Hagen, 1967; McPhail, 1994; Jones *et al.*, 2006). With this scenario, I have different possible interpretation for the actual ecomorph diversity. The first one is that the low-plated ecomorph found in Lough Furnace originated from the ancestral freshwater population at some time in the distant past, but has not diverged morphologically from it to any great extent. In this case, it is possible that they might have diverged genetically and form different gene pools, but have converged to the same phenotype. The second one is that the low-plated individuals derive from the contemporary freshwater population and are constantly being recruited from that source, maintaining a downstream gene flow between them.

Potential pre-zygotic reproductive barriers and potential selection against hybrids?

Ravinet *et al.*, (2015) showed that there was little evidence for hybridisation in Lough Furnace stickleback ecomorphs with microsatellites markers and recommended the study of various potential reproductive barriers that could reduce gene flow between ecomorphs to account for their finding (i.e., habitat isolation, allochrony and mate choice). In this study, I explored some of the reproductive barriers that could potentially play a role in Lough Furnace.

Allochrony, a mismatch in breeding time between two populations that reduces gene flow, is known to occur in some systems, even partially with early spawning populations and late spawning populations. Here, I showed that sexually mature individuals – i.e. gravid females and males in breeding colours – were present in the same locations in Lough Furnace and at the same time. Other studies have shown that allochrony had played a role in reproductive isolation among stickleback populations in sympatry (Hagen, 1967). Hanson *et al.*, (2016a) showed in a comparison of multiple paired stream-lake populations that allochrony could potentially explain differences observed between lake and stream dwelling stickleback ecomorphs, but its role in preventing gene flow was likely to be weak and that reproductive isolation is usually reinforced by other barriers.

Habitat isolation and mate choice have been proposed previously as to be strong pre-zygotic barrier to gene flow among various ecomorph pairs (e.g., limnetic-benthic, anadromous-freshwater, lake-stream) (Lackey & Boughman, 2017). For example, in respect of habitat isolation, Kume *et al.*, (2010) and Ravinet *et al.*, (2021) showed that two anadromous stickleback species, one overwintering in the Pacific Ocean (*G. aculeatus)* and the other in the Japan sea (*G. nipponicus)*, while co-occurring in several Japanese rivers were observed to spawn predominantly at different locations. Those

originating in the Japan Sea spawn in the estuary, while the Pacific Ocean stickleback breed mainly further upstream in freshwater. Nevertheless, there was still evidence between both for a low level of interbreeding. In Lough Furnace, the data would suggest that habitat isolation does not have a strong impact as I observed both ecomorphs at the same sampling sites during the breeding season. However, the nature of habitat isolation can be very complex as Bentzen *et al.*, (1984) showed among benthic-limnetic pairs occupying different habitats. They found in a study of stickleback populations in Enos Lake, Vancouver Island, British Columbia, that limnetic males came to the shore to build their nests, at the same locations as the benthic males. Meanwhile, limnetic females remain segregated from the others, and only come near the shore for a short time to breed with limnetic males. Even if both sexes and ecomorphs overlap at the same place on the shoreline, mate choice still occurs, especially for limnetic females and males.

Despite a low sample size and probable biases in mortality among mesocosms, I were able to show here that hybridisation can occur between ecomorphs even if potentially only in one direction (completely-plated females with low-plated males). Several reasons could account for the apparent absence of hybrids reported by Ravinet *et al.* (2015) and in this study. Practically, in my breeding experiments, the main challenge was to adjust successfully all fish (and particularly high plated morphs) to the freshwater conditions in the breeding mesocosms. Justifying this approach Defaveri & Merilä (2014) and Divino *et al.*, (2016) showed that all juvenile fish, regardless of the salinity in which they originated, have better performance in low-salinity environment than in an high-salinity environment (higher juvenile survival, better growth, better body condition). However, contrary to expectation based on these studies above, the completely-plated males did not appear to perform well in freshwater and to succeed in breeding, probably due to the fact that in the freshwater mesocosms, completely-plated males were not in optimal

condition, affecting their nest quality and level of production (Barber *et al.*, 2001). What one might expect theoretically, is that mate choice is an important factor underpinning reproductive isolation and even if different ecomorphs can interbreed, it does not mean that mate choice is absent. For example, male courtship behaviour can be lineage specific (Kitano *et al.*, 2007). Males can adapt their courtship behaviour depending on the female's ecomorph (McPhail & Hay, 1983; Ridgway & McPhail, 1984; Blouw D. M. & Hagen, 1990). Ecomorph specific behaviour can also evolve quickly as Ziuganov (1995) showed that eight generations were sufficient to develop a behavioural reproductive barrier between completely-plated and low-plated. Like shown in this study, reproduction can be in one direction (Kitano *et al.*, 2009). During their interspecific laboratory mate choice trials, Kitano *et al.*, (2009) showed that Pacific Ocean females mate exclusively with Pacific Ocean males, while Japan Sea females mate with both types of males, with the hybrid males produced having substantially reduced fertility. In an intraspecific study, Jones *et al.*, (2006) showed that while there was no apparent reason for a directional bias in hybridisation, those hybrids found had more frequently an anadromous mother, probably due to the relative numbers of freshwater and anadromous females at spawning time. This might also be important in Lough Furnace where completely-plated ecomorphs (both males and females) are less abundant than low-plated, therefore, reducing the likelihood of producing hybrids. In these breeding experiments, the number of completely-plated individuals that could be introduced into the mesocosms was limiting, as they were captured less often than low-plated. It is reasonable to assume that mate choice is an important driver of reproductive isolation, but when no choice is offered, different ecomorphs do interbreed, even though they may well have a greater preference for a mate of their own kind (Ridgway & McPhail, 1984). The potential for mate choice was not tested in this breeding mesocosms because of the

limited number of both female and male completely-plated individuals available. Ideally, a fifth tank with a mixture of females and males of both ecomorphs would have provided an opportunity to test for mate choice. Due to the different experimental protocol limits, especially due to the absence of salinity and temperature control, I observed a high stress level particularly in completely-plated individuals, and a high mortality rate. For this reason, no strong conclusions can be made about the hybridisation between the two ecomorphs in the wild.

In addition to the genetic analyses already undertaken by Ravinet *et al*. (2015), other possible genetic investigations would be indicative: (1) to provide more powerful markers for the detection of hybrids, (2) to resolve colonisation history of stickleback in the Burrishoole system, and (3) to identify the regions of the genomic divergence that might underpin local adaptation and ultimately sympatric speciation. In respect of the third objective (Guo *et al.*, 2015) showed that different stickleback ecomorphs sampled across a salinity gradient diverged significantly among genomic regions harbouring important gene functions linked with the immune system. These were probably under balancing selection and associated with selective pressures in the freshwater environment (Ishikawa *et al.*, 2019). Genomic analysis would also shed light on the potential presence of multigenerational hybrids living in the system and their proportions. For example, in one study of benthic-limnetic pairs, selection against hybrids seemed very weak or non-existing (Hanson *et al.*, 2016b) but, in other studies habitat preference was found to have an important role in selecting against hybrids (Bolnick *et al.*, 2009; Jiang *et al.*, 2015). Studies on benthic-limnetic stickleback pairs showed that hybrids have an inferior growth rate (Taylor *et al.*, 2012) and their frequency decreased during the life cycle, suggesting selection against them (Gow *et al.*, 2007).

Ravinet *et al.* (2015) concluded based on genetic analyses that only a small amount of gene flow as likely to be ongoing between the divergent freshwater and anadromous forms in Lough Furnace. I found, that currently at least, there were no spatial, temporal or maturity-scheduling obstacles to gene flow between ecomorphs to explain why this might occur. Furthermore, I know hybrids are a possibility from the breeding experiments, but that they probably occur at low rates. On the basis of what possibilities are left, it would appear that mate choice is likely to have an important part to play and an important candidate for further research. Alternately and possibly in conjunction, disruptive selection could also contribute to maladapted intermediate ecomorphs. The phenotypic variation within this system as was previously suggested by Ravinet *et al*. (2015) may be underpinned by a more complex genetic architecture than might be first appreciated. However, I would be less certain as to the contribution of any potential genetic incompatibilities in preventing or limiting the occurrence of hybrids. Future genomic studies could focus on obtaining a greater understanding of the genes underlying morphological diversity and hence indirectly the mechanisms responsible for driving adaptive divergence and limiting gene flow between freshwater resident and anadromous stickleback forms.

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Supplementary material

S2.1. Fish care and welfare.

The diet of wild sticklebacks consists of microcrustaceans such as copepods, cladocerans and ostracods, the larvae and the pupae of the chironomids and/or aquatic nymphs of mayflies (Wootton, 1984). They can also feed on oligochaetes, small molluscs and algae. In this experiment, tanks were supplied with continuous water flow coming from Lough Feeagh with no filtration system. The water was not treated and would contain all the crustaceans, cladocerans, aquatics nymphs and algae present (de Eyto *et al.*, 2016). Additional food was allotted to every tank to boost adult reproductive status and juvenile growth. Females can have larger and more numerous clutches if food is abundant (Wootton, 1984). The food was given in small amounts across the tank to prevent any competition to access the resources. To ensure *ad libidum* feeding, fish would be observed while food is distributed to note how fast they are reaching the food and if there is any food left after feeding. The amount of food given was adjusted to the density per tank. To stimulate fish foraging behaviour and complement the diet, 20g of live bloodworms and 50 grams of GoldPearls (JBL GmbH & Co. KG, Germany) would be given three times a week.

Table S2.2. Summary of the main sampling location and different sites with their different parameters: Lat : latitude, Long : longitude, Temperature: temperature recorded at the sampling site, Salinity: salinity temperature recorded at the sampling site, TotalFish: total number of fish caught a this sampling site, Released: number of fish released after counting, Collected : number of fish kept.

Date	Month	Year	Location	Site	Lat	Long	Temperature	Salinity	TotalFish	Released	Collected
08/05/2018	May	2018	FEE	SNB	53.95652	-9.57157	14	0	0	0	0
08/05/2018	May	2018	FEE	TH	53.94973	-9.56702	14.3	0	4	0	4
08/05/2018	May	2018	FEE	EB	53.93952	-9.5688	12.1	0	2	0	2
08/05/2018	May	2018	FEE	TB	53.93954	-9.56875	12	0	0	0	0
13/01/2018	April	2018	FUR	AB	53.89865	-9.57347	11.1	NA	18	0	18
09/05/2018	May	2018	FUR	AB	53.89865	-9.57347	10.2	NA	12	0	12
09/05/2018	May	2018	FUR	Shed	53.90492	-9.57766	10.2	NA	3	0	3
09/05/2018	May	2018	FUR	7A	53.90392	-9.5749	9.5	NA	4	0	4
09/05/2018	May	2018	FUR	NB	53.90659	-9.57363	9.2	NA	8	0	8
10/05/2018	May	2018	FUR	MI	53.92222	-9.57351	12.6	2.6	21	0	21
10/05/2018	May	2018	FUR	SL	53.91917	-9.58318	12.6	1.9	4	0	4
10/05/2018	May	2018	FUR	Slo	53.91561	-9.58391	13	4.4	24	0	24
10/05/2018	May	2018	FUR	NP	53.91255	-9.58032	14.3	5.1	40	0	40
10/05/2018	May	2018	FUR	SP	53.91196	-9.58025	16	4.8	8	0	8
20/06/2018	June	2018	FUR	AB	53.89865	-9.57347	14	0.9	0	0	0
20/06/2018	June	2018	FUR	Shed	53.90492	-9.57766	14.2	7.6	10		10
20/06/2018	June	2018	FUR	7A	53.90392	-9.5749	14.5	9.7	0	0	0
20/06/2018	June	2018	FUR	NB	53.90659	-9.57363	14.1	6.1	10		10
21/06/2018	June	2018	FEE	SNB	53.95652	-9.57157	12.6	0	0		0
21/06/2018	June	2018	FEE	TH	53.94973	-9.56702	15.4	0	3		3
21/06/2018	June	2018	FEE	EB	53.93952	-9.5688	14	0	0		0
21/06/2018	June	2018	FEE	TB	53.93954	-9.56875	14.8	0	0		0
22/06/2018	June	2018	FUR	MI	53.92222	-9.57351	17.4	1.8	10		10

22/06/2018	June	2018	FUR	SL	53.91917	-9.58318	17.6	0.1	0		0
22/06/2018	June	2018	FUR	Slo	53.91561	-9.58391	14.8	2.3	10		10
22/06/2018	June	2018	FUR	NP	53.91255	-9.58032	16.8	3.7	6		6
22/06/2018	June	2018	FUR	SP	53.91196	-9.58025	15.6	6	10		10
22/06/2018	June	2018	FUR	AB	53.89865	-9.57347	13.2	1.8	10		10
18/07/2018	July	2018	FUR	SP	53.91196	-9.58025	15.8	10.1	0		0
18/07/2018	July	2018	FUR	NP	53.91255	-9.58032	16.8	10.1	15		15
18/07/2018	July	2018	FUR	Slo	53.91561	-9.58391	17.6	11.2	0		0
18/07/2018	July	2018	FUR	SL	53.91917	-9.58318	17.7	1.5	7		7
18/07/2018	July	2018	FUR	MI	53.92222	-9.57351	18.2	1.1	8		8
19/07/2018	July	2018	FUR	AB	53.89865	-9.57347	17.4	17.8	1		1
19/07/2018	July	2018	FUR	Shed	53.90492	-9.57766	18.5	14.6	0		0
19/07/2018	July	2018	FUR	7A	53.90392	-9.5749	18.5	18.9	0		0
19/07/2018	July	2018	FUR	NB	53.90659	-9.57363	18.2	11	22		22
20/07/2018	July	2018	FEE	SNB	53.95652	-9.57157	18.1	0	0	0	0
20/07/2018	July	2018	FEE	TH	53.94973	-9.56702	18.5	0	1	0	1
20/07/2018	July	2018	FEE	EB	53.93952	-9.5688	18.2	0	3	0	3
20/07/2018	July	2018	FEE	TB	53.93954	-9.56875	18.3	0	0	0	0
26/07/2018	July	2018	FEE	NWRB	53.95379	-9.58225	19.04	0	0	0	0
26/07/2018	July	2018	FEE	WEB	53.94357	-9.58201	18.73	0	0	0	0
26/07/2018	July	2018	FEE	WTB	53.93574	-9.58339	18.5	0	0	0	0
26/07/2018	July	2018	FEE	RH	53.92304	-9.58585	18.14	0	4	0	4
26/07/2018	July	2018	FEE	Russel	53.9517	-9.58823	NA	0	5	0	5
29/08/2018	August	2018	FUR	Shed	53.90492	-9.57766	14.7	3.8	10		10
29/08/2018	August	2018	FUR	NB	53.90659	-9.57363	14.5	1	1		1
29/08/2018	August	2018	FUR	7A	53.90392	-9.5749	15.4	3.9	1		1
29/08/2018	August	2018	FUR	AB	53.89865	-9.57347	15.55	5.8	1		1
30/08/2018	August	2018	FEE	RH	53.92304	-9.58585	14.9	0	0	0	0

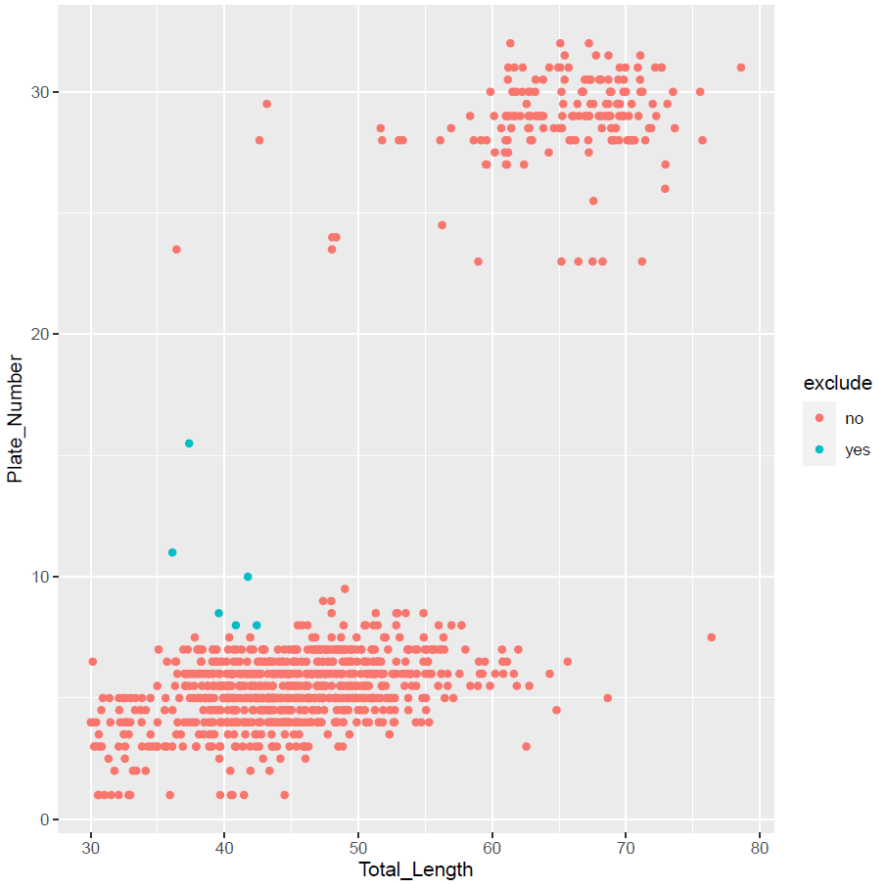
30/08/2018	August	2018	FEE	WTB	53.93574	-9.58339	14.7	0	0	0	0
30/08/2018	August	2018	FEE	WEB	53.94357	-9.58201	14.5	0	0	0	0
30/08/2018	August	2018	FEE	NWRB			14.57	0	0	0	0
30/08/2018	August	2018	FEE	TH	53.94973	-9.56702	15.2	0	22	0	22
30/08/2018	August	2018	FEE	EB	53.93952	-9.5688	14.7	0	0	0	0
30/08/2018	August	2018	FEE	TB	53.93954	-9.56875	14.4	0	0	0	0
31/08/2018	August	2018	FUR	SP	53.91196	-9.58025	14.5	1.4	11		11
31/08/2018	August	2018	FUR	NP	53.91255	-9.58032	NA	NA	0		0
31/08/2018	August	2018	FUR	Slo	53.91561	-9.58391	NA	NA	2		2
31/08/2018	August	2018	FUR	SL			NA	NA	5		5
31/08/2018	August	2018	FUR	MI	53.92222	-9.57351	NA	NA	6		6
20/04/2018	April	2018	FUR	NB	53.90659	-9.57363			34		34
18/04/2018	April	2018	FUR	Shed	53.90492	-9.57766			26		26
04/04/2018	April	2018	FUR	Russel	53.9517	-9.58823			1		1
10/05/2018	May	2018	FUR	Russel	53.9517	-9.58823			10		10
20/06/2018	June	2018	FUR	Russel	53.9517	-9.58823			13		13
10/07/2018	July	2018	FUR	Russel	53.9517	-9.58823			45		45
16/01/2019	January	2019	FUR	MI	53.92222	-9.57351	NA	NA	21	0	21
16/01/2019	January	2019	FUR	SL	53.91917	-9.58318	NA	NA	9	1	8
16/01/2019	January	2019	FUR	Slo	53.91561	-9.58391	NA	NA	56	11	45
16/01/2019	January	2019	FUR	NP	53.91255	-9.58032	NA	NA	5	0	5
16/01/2019	January	2019	FUR	SP	53.91196	-9.58025	NA	NA	0	0	0
17/01/2019	January	2019	FEE	SNB	53.95652	-9.57157	NA	NA	0	0	0
17/01/2019	January	2019	FEE	TH	53.94973	-9.56702	NA	NA	0	0	0
17/01/2019	January	2019	FEE	EB	53.93952	-9.5688	NA	NA	0	0	0
17/01/2019	January	2019	FEE	TB	53.93954	-9.56875	NA	NA	0	0	0
17/01/2019	January	2019	FUR	AB	53.89865	-9.57347	NA	NA	3	0	3
17/01/2019	January	2019	FUR	Shed	53.90492	-9.57766	NA	NA	15	0	15

17/01/2019	January	2019	FUR	7A	53.90392	-9.5749	NA	NA	7	0	7
17/01/2019	January	2019	FUR	NB	53.90659	-9.57363	NA	NA	1	0	1
08/04/2019	April	2019	FUR	Slo	53.91561	-9.58391	10.5	0.2	14	0	14
08/04/2019	April	2019	FUR	Shed	53.90492	-9.57766	10.8	4.7	3	0	3
10/04/2019	April	2019	FUR	Slo	53.91561	-9.58391	14.1	0.2	15	0	15
10/04/2019	April	2019	FUR	Shed	53.90492	-9.57766	13.1	5	23	1	22
23/04/2019	April	2019	FUR	AB	53.89865	-9.57347	13.5	21.3	11	0	11
23/04/2019	April	2019	FUR	Shed	53.90492	-9.57766	13	9.9	40	21	19
23/04/2019	April	2019	FUR	7A	53.90392	-9.5749	13.1	18.3	55	52	3
23/04/2019	April	2019	FUR	NB	53.90659	-9.57363	12.9	7.4	6	0	6
24/04/2019	April	2019	FUR	MI	53.92222	-9.57351	12.3	3.5	11		11
24/04/2019	April	2019	FUR	SL	53.91917	-9.58318	12.3	0	36	27	9
24/04/2019	April	2019	FUR	Slo	53.91561	-9.58391	11.1	12.2	70	54	16
24/04/2019	April	2019	FUR	NP	53.91255	-9.58032	5.7	13.7	4	0	4
24/04/2019	April	2019	FUR	SP	53.91196	-9.58025	6.3	13	2	0	2
25/04/2019	April	2019	FEE	SNB	53.95652	-9.57157	13.1	0	0	0	0
25/04/2019	April	2019	FEE	TH	53.94973	-9.56702	13.4	0	6	0	6
25/04/2019	April	2019	FEE	EB	53.93952	-9.5688	12.2	0	3	0	3
25/04/2019	April	2019	FEE	TB	53.93954	-9.56875	12.6	0	0	0	0
30/04/2019	April	2019	FUR	Slo	53.91561	-9.58391	13.8	9.7	27	22	5
14/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	18.9	5.7	8	4	4
14/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	12.6	2.6	4	2	2
16/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	15.3	7.77	13	7	6
21/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	13.1	8.9	0	0	0
22/05/2019	May	2019	FUR	MI	53.92222	-9.57351	14.5	4.5	1	1	0
22/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	15.4	16.3	6	6	0
23/05/2019	May	2019	FUR	MI	53.92222	-9.57351	15.9	10.8	18	18	0
24/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	15.9	15.3	34	34	0

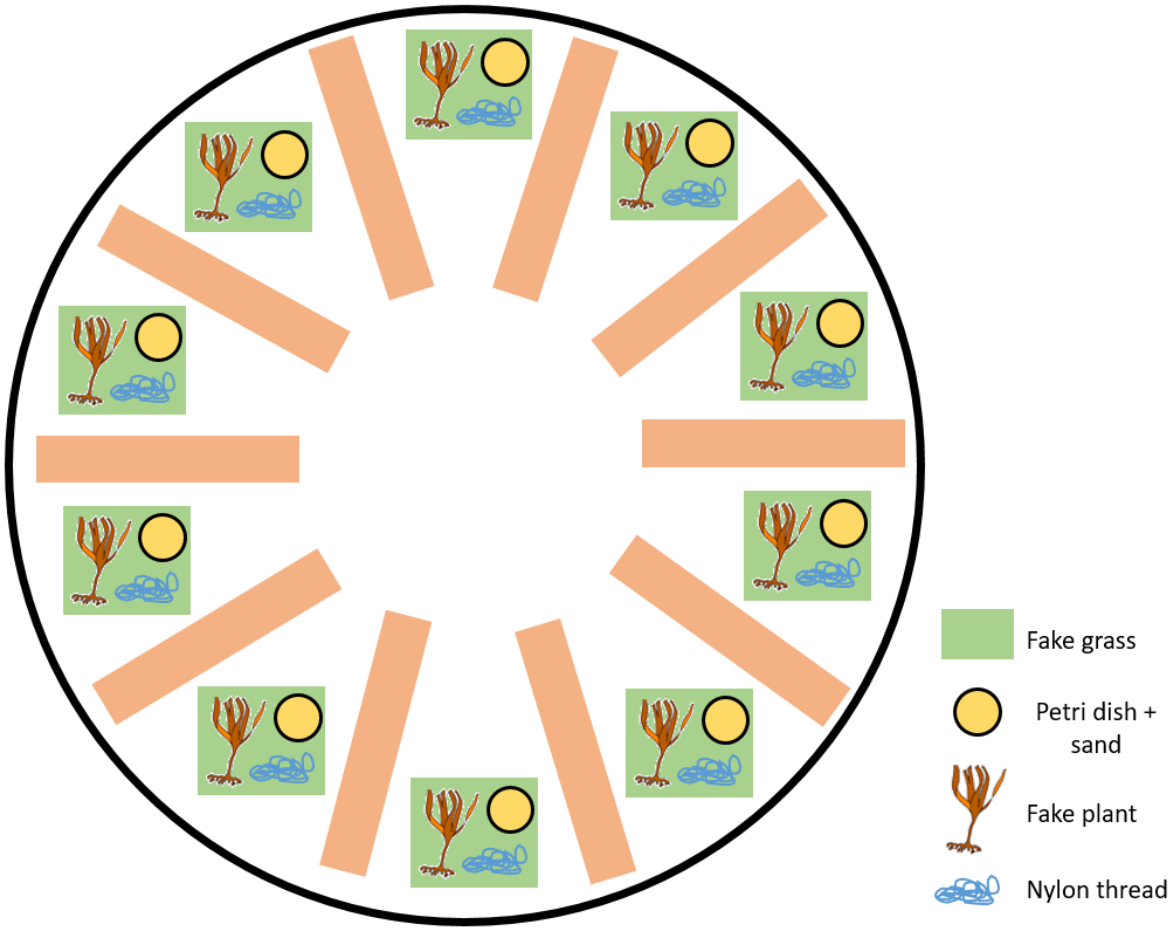
24/05/2019	May	2019	FUR	MI	53.92222	-9.57351	15.6	10.4	0	0	0
25/05/2019	May	2019	FUR	MI	53.92222	-9.57351	14.1	5.3	22	22	0
27/05/2019	May	2019	FUR	MI	53.92222	-9.57351	14.4	10	5	5	0
27/05/2019	May	2019	FUR	Slo	53.91561	-9.58391	15.8	16.5	1	1	0
04/06/2019	June	2019	FEE	TH	53.94973	-9.56702	NA	0	6		6
04/06/2019	June	2019	FEE	SNB	53.95652	-9.57157	NA	0	0		0
05/06/2019	June	2019	FUR	SP	53.91196	-9.58025	15.2	3.1	49	37	12
05/06/2019	June	2019	FUR	NP	53.91255	-9.58032	15.3	1.8	41	31	10
05/06/2019	June	2019	FUR	SL	53.91917	-9.58318	15.9	0	27	17	10
05/06/2019	June	2019	FUR	Slo	53.91561	-9.58391	13.6	0.7	0	0	0
05/06/2019	June	2019	FUR	MI	53.92222	-9.57351	13.9	1.1	13	6	7
06/06/2019	June	2019	FUR	Shed	53.90492	-9.57766	13.1	18.7	28	11	17
06/06/2019	June	2019	FUR	7A	53.90392	-9.5749	13.2	19.9	22	11	11
06/06/2019	June	2019	FUR	NB	53.90659	-9.57363	12.5	5.06	28	14	14
06/06/2019	June	2019	FUR	AB	53.89865	-9.57347	13.6	5.7	0	0	0
20/06/2019	June	2019	FUR	NB	53.90659	-9.57363	15.9	2.6	41	28	13
20/06/2019	June	2019	FUR	Shed	53.90492	-9.57766	16.2	4.9	10	8	2
24/06/2019	June	2019	FUR	SP	53.91196	-9.58025	17.4	3.9	61	47	14
24/06/2019	June	2019	FUR	NP	53.91255	-9.58032	17	2.5	23	10	13
25/06/2019	June	2019	FUR	SP	53.91196	-9.58025	17.5	6.4	7		7
26/06/2019	June	2019	FUR	SP	53.91196	-9.58025	20.4	0.8	10	5	5
27/06/2019	June	2019	FUR	Shed	53.90492	-9.57766	17.6	4.4	4	2	2
28/06/2019	June	2019	FUR	Shed	53.90492	-9.57766	18.5	4.4	1	1	0
30/06/2019	June	2019	FUR	Shed	53.90492	-9.57766	17.7	5.1	17	9	8
16/07/2019	July	2019	FUR	SP	53.91196	-9.58025	21.9	9.1	13	13	0
17/07/2019	July	2019	FUR	SP	53.91196	-9.58025	17.3	9	0		0
17/07/2019	July	2019	FUR	NP	53.91255	-9.58032	17.6	7.5	6		6
17/07/2019	July	2019	FUR	Slo	53.91561	-9.58391	18.4	14.3	2		2

17/07/2019	July	2019	FUR	SL	53.91917	-9.58318	18.7	0	5		5
17/07/2019	July	2019	FUR	MI	53.92222	-9.57351	18	0	0		0
18/07/2019	July	2019	FUR	AB	53.89865	-9.57347	19	15.7	16	15	1
18/07/2019	July	2019	FUR	Shed	53.90492	-9.57766	NA	NA	1	0	1
18/07/2019	July	2019	FUR	7A	53.90392	-9.5749	199	17.8	0	0	0
18/07/2019	July	2019	FUR	NB	53.90659	-9.57363	18.4	7.9	9	9	0
19/07/2019	July	2019	FEE	SNB	53.95652	-9.57157	16.5	0	0	0	0
19/07/2019	July	2019	FEE	TH	53.94973	-9.56702	17.4	0	4	0	4
19/07/2019	July	2019	FEE	TB	53.93954	-9.56875	17.3	0	0	0	0
19/07/2019	July	2019	FEE	EB	53.93952	-9.5688	17.2	0	1	0	1
24/07/2019	July	2019	BEL	RP1	54.10269	-9.99163	18.3	31.4	110	?	110
13/08/2019	August	2019	BEL	RP1	54.10269	-9.99163	17.5	25.1	21	?	21
28/08/2019	August	2019	FUR	AB	53.89865	-9.57347	17.2	1.3	6	6	0
07/10/2019	October	2019	FUR	Shed	53.90492	-9.57766			19		19
07/10/2019	October	2019	FUR	NB	53.90659	-9.57363			13		13
04/06/2019	June	2019	FEE	EB					2		2
04/06/2019	June	2019	FEE	RB					1		1
19/07/2019	July	2019	FEE	Russel	53.9517	-9.58823			3	0	3
23/03/2019	March	2019	FUR	7A	53.90392	-9.5749	13.1	18.3	3		3
	February	2019	FUR	7A	53.90392	-9.5749	13.1	18.3	0	0	0
28/08/2019	August	2019	FUR	MI	53.92222	-9.57351			2		2
28/08/2019	August	2019	FUR	NB	53.90659	-9.57363			14		14
28/08/2019	August	2019	FUR	NP	53.91255	-9.58032			1	0	1
12/03/2019	March	2019	FUR	Shed	53.90492	-9.57766			20		20
11/03/2019	March	2019	FUR	Slo	53.91561	-9.58391			10		10
28/08/2019	August	2019	FUR	SP	53.91196	-9.58025			2		2
18/05/2020	May	2020	FUR	MI	53.92222	-9.57351	14.8	14.9	11	NA	11
18/05/2020	May	2020	BEL	RP1	54.10269	-9.99163	13.3	35.9	34		34

19/05/2020	May	2020	FUR	Slo	53.91561	-9.58391	13.7	18.4	42	NA	42
19/05/2020	May	2020	FUR	7A	53.90392	-9.5749	13.9	20.7	44	NA	44
20/05/2020	May	2020	FUR	MI	53.92222	-9.57351	13.8	1.8	2	NA	2
21/05/2020	May	2020	FUR	Slo	53.91561	-9.58391	14.4	18.3	4	NA	4
21/05/2020	May	2020	FUR	7A	53.90392	-9.5749	16.1	21.3	13	NA	13
22/05/2020	May	2020	FEE	TH	53.94973	-9.56702	11.3		10		10
22/05/2020	May	2020	FEE	EB	53.94973	-9.56702	11.3		6		6
22/05/2020	May	2020	FUR	7A	53.90392	-9.5749	13.4	28.5	4	NA	4
24/05/2020	May	2020	FUR	7A	53.90392	-9.5749	13.5	17.2	15	NA	15
24/05/2020	May	2020	FUR	7A	53.90392	-9.5749	13.8	16	NA	NA	x



S2.3. Outliers identification: outliers were identified by eye observation as they were completely outside the distribution. I removed from the dataset individuals that total length was inferior to 45 mm and their total plate number superior to 7. Distribution of the number of plate according to the total length with the six outliers (in blue) and all the other individuals (in red).



S2.4. Diagram of the tank design with enrichments.

S2.5. Picture of the setup in one tank.

S2.6. Summary of the sampling sites and their caracteristics information for the fish used in the breeding experiment. Lat: latitude, long: longitude, Mean_salinity: mean salinity calculated from all the salinity recorded, Min_salinity: the minimum salinity measured at each site, Max_salinity: the maximum salinity measured at each site.

Location	Site	Lat	Long	Mean_salinity	Min_salinity	Max_salinity
FUR	MI	53.92222	-9.57351	6.2	0	14.9
FUR	SP	53.91196	-9.58025	6.5	0.8	13
FUR	Slo	53.91561	-9.58391	9.8	0.2	18.4

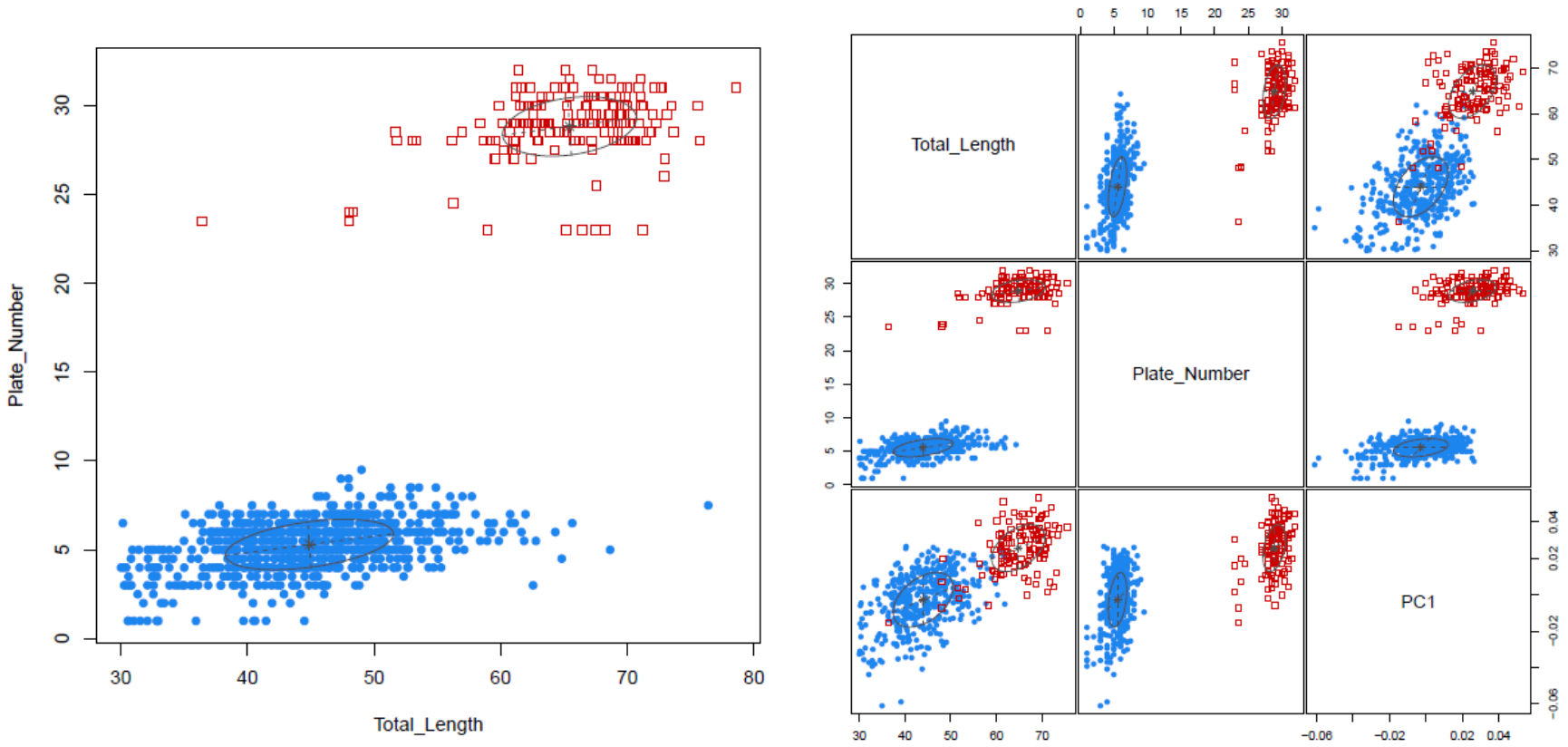
S2.7. Breeding experiment information. CP = completely-plated; LP = low-plated; F = Female; M = Males; Nest = number of nests produced by males in this tank; Juvenile = number of juveniles produced at the end of the experiment.

Tank	Crossing	Nest	Juvenile
1	CP_F x LP_M	8	29
2	LP_F x LP_M	5	21

3	CP_M X LP_F	3	0
4	CP_F X CP_M	0	0

S2.8. Summary of the four best models of clustering analysis for all individuals (MU and BUR) but with no morphometry. K= number of clusters; Type = type of models; BIC = Bayesian information criterion.

Order	K	Type	BIC
1	2	EVE	-10665.8
2	2	VVE	-10667.4
3	3	VVE	-10669.9
4	2	EVV	-10669.9



S2.9. Distribution of the two clusters in the first model with all individuals from Muingdoran and Burrishoole (n= 974) with total length and the lateral plate number. b) Distribution of the two clusters in the second model with all individuals from Muingdoran and Burrishoole (n= 533) with total length, the lateral plate number and PC1 (first morphometric component).

S2.10. Summary of the four best models of clustering analysis for all individuals (MU and BUR) with morphometry. K= number of clusters; Type = type of models; BIC = Bayesian information criterion.

Order	k	Type	BIC
1	2	EVE	-2792.9
2	2	VVE	-2798.3
3	2	EEE	-2805.9
4	3	EVE	-2807.6

S2.11. Summary of the four best models of clustering analysis for individuals in Lough Furnace but without morphometry. K= number of clusters; Type = type of models; BIC = Bayesian information criterion.

Order	k	Type	BIC
1	2	EVV	-8616.5
2	2	EEV	-8620.4
3	2	VVV	-8620.8
4	2	VEV	-8624.0

S2.12. Summary of the four best models of clustering analysis for individuals in Lough Furnace with morphometry. K= number of clusters; Type = type of models; BIC = Bayesian information criterion.

Order	k	Type	BIC
1	2	VVE	-2363.000658
2	2	EVE	-2370.272206
3	3	VVE	-2370.747359
4	2	VVV	-2371.714717

S2.13. Summary table of individuals introduced in the breeding experiment, the number of death and cumulative death calculated.

Ecomorph	Introduced	Mort	FinalCumulatedDeath
HP_F	30	30	100
LP_F	44	29	65.9
HP_M	14	14	100
LP_M	35	26	74.3

Chapter 3: Genetic structure in different stickleback (*Gasterosteus aculeatus*) ecomorphs living in parapatry and sympatry in the Burrishoole system western Ireland

Abstract

Understanding patterns of parallel evolution at the intraspecific level, and the genomic and ecological mechanisms involved, is fundamental in evolutionary biology. This study aimed to characterise patterns of neutral and adaptive genetic divergence among sticklebacks (*Gasterosteus aculeatus*) in an ecologically-complex system in the west of Ireland, grouped *a priori* into three putative ecotypes: low-plated (LP) individuals sampled in freshwater, LP individuals sampled in a brackish lagoon, and completely-plated (CP) individuals sampled in the same brackish lagoon. Two additional groups of CP individuals sampled from fully marine environments outside the study catchment (one from geographically proximate location in coastal NW Ireland, one from the Hebrides, Scotland) were also included, to help separate the effects of morphotype (LP vs CP) from salinity niche (freshwater, brackish, marine). 1,599 individuals (sampled over a period of ten years and three main locations) were successfully genotyped at two different classes of SNPs obtained from the stickleback literature: 10 putatively functional SNPs that were shown previously to consistently differentiate marine versus freshwater population pairs across a broad geographic range, and 22 putatively neutral SNPs that showed no signs of consistent divergent selection between marine versus

freshwater population pairs. The results of the current study showed that the putatively neutral SNPs primarily differentiated freshwater from brackish/marine groups, with the marine samples being genetically more similar to the brackish samples than the freshwater samples, suggesting a scenario of isolation-by-environment rather than isolation-by-distance**.** The putatively adaptive markers, in contrast, primarily differentiated LP from CP individuals, with a second minor axis of variation differentiating individuals more by the salinity of the environment from which they were collected. When analysing loci separately, however, it became apparent that the overall patterns for the 10 putatively functional markers were driven by two SNPs in particular (associated with salinity tolerance and metabolism genes), while the other eight SNPs (found to be associated with a range of other functions in other studies) primarily differentiated samples based on salinity environment and thus the patterns were more similar to those exhibited by the putatively neutral loci. Taken together, these findings imply a limited amount of parallelism at the SNP level with respect to morphotypes (given that only 2 of 10 putatively adaptive SNPs clearly differentiated LP from CP individuals), and that isolation-by-adaptation with respect to salinity environment might indirectly shape patterns of neutral divergence. Other genomic approach would better characterise adaptive and neutral genetic divergence in this system, and how each in turn maps onto morphological, physiological and behavioural differences among putative ecotypes.

Keywords: Sympatric speciation, isolation-by-adaptation, salinity tolerance, stickleback

Introduction

Understanding genetic variability in natural populations through time and space, and the ecological and genomic factors involved in structuring and sustaining this variation, is central to the exploration the processes underpinning the study of evolution (Rundle & Nosil, 2005). When populations colonise new environments, natural and sexual selection, adaptive divergence, and neutral processes such as genetic drift associated with variations in population size can all contribute to the development of reproductive barriers which ultimately generate within a species distinct new genetic groups or populations and with time potentially develop into new species, (Schluter, 2000b; Berner *et al.*, 2009; Jones *et al.*, 2012c). Understanding the standing genetic basis of adaptation is important, as adaptation from *de novo* mutations is likely to be slower than adaptation from standing variation (Barrett & Schluter, 2008). Studying contact zones, where different populations or species overlap, can highlight the roles of gene flow and hybridisation in facilitating or restricting adaptive divergence. These transition zones are often found across environmental gradients, where individuals can settle in different ecological niches (Barton & Hewitt, 1985; Hendry *et al.*, 2009).

Adaptive divergence within species can maintain functional genetic polymorphism, and may contribute to the development of genome-wide reproductive barriers (Payseur, 2010). However, the implications of gene flow for population fitness are variable and context-specific. On one hand, high gene flow may slow or prevent the process of local adaptation (García-Ramos & Kirkpatrick, 1997; Kirkpatrick & Barton, 1997). Conversely, it may also facilitate shifts from one adaptive peak to another (Peck et al., 1998). Numerous studies, on a diverse array of taxa, have documented adaptive divergence between population pairs under divergent selection. However, when

populations co-occur in a contact zone, they have the opportunity to hybridize. Examples include interspecies such as *Passerina amoena* and *Passerina cyanea* in birds (Carling & Brumfield, 2009) or the house mice (*Mus musculus* and *Mus domesticus)* (Teeter *et al.*, 2008). Examples in a contact zone, in parapatry or sympatry, include a wide range of taxa such as amphibians with the fire- and yellow-bellied toads (*Bombina bombina* and *B. variegata*) (Dufresnes *et al.*, 2020) or fish, sticklebacks (*Gasterosteus aculeatus*) (Lavin & McPhail, 1986), coho salmon (*Oncorhynchus kisutch*) (Swain & Holtby, 1989), sockeye salmon (*Oncorhynchus nerka*) (Hendry et al., 2000) or kokanee (*Oncorhynchus nerka*) (Taylor et al., 1997). Therefore, understanding and quantifying gene flow in populations co-existing in contact zones has the opportunity to illuminate how genetic diversity is maintained.

Spatial and temporal processes play important roles in shaping genetic differentiation between natural populations. Understanding how these processes can influence gene flow between and among populations and, consequently, interspecific genetic variation, is a subject of ongoing intensive study in evolutionary biology and ecology (Wright, 1943; Nosil, 2012; Balkenhol *et al.*, 2015). Several processes can contribute to genetic differentiation between different populations, including physical isolation, whereby genetic divergence increases as geographic distance increases (Wright, 1943), and ecological isolation, whereby different mechanisms reduce gene flow between different environments through divergent selection (Wang & Bradburd, 2014). Both physical isolation (isolation-by-distance, IBD) and ecological isolation (isolation-by-environment, IBE) are likely to facilitate the fixation of adaptive alleles and therefore, drive local adaptation in natural populations (Nosil, 2012). However, when adaptive divergence promotes reproductive barriers, and as a consequence, reduces gene flow between population from ecologically divergent environments; genetic differentiation at

neutral loci (not under selective divergence) increases as well, and this process is isolation-by-adaptation (IBA) (Nosil *et al.*, 2009; Orsini *et al.*, 2013). Understanding the evolutionary mechanisms that shape intraspecific variation requires disentangling these processes (Wang *et al.*, 2013; Balkenhol *et al.*, 2015).

Temporal variation is also important in studying the phenotypic variation within populations and, their ability to adapt to local conditions and for ecological speciation (Sasaki & Ellner, 1997; Bell, 2010; Svardal *et al.*, 2011). Different temporal scales, from seasonal shifts of a few months duration to a few thousand years when colonizing a new environment, can drive phenotypic changes (Nosil, 2012). For example, some studies have observed that seasonal shifts can promote directional selection in the plate number of three-spined sticklebacks, between summer and winter depending on the type and magnitude of pressure from predators (Reimchen, 1995, 2000; Reimchen & Nosil, 2004). In guppies (*Poecilia reticulata*), the well-characterised spatially-variable selection pressures acting on male coloration are also known to be subject to strong temporal variation (Gotanda & Hendry, 2014).

The three-spined stickleback is a small teleost fish and a model species in evolutionary biology studies, known for showing extensive morphological, ecological and genetic variation (Wootton, 1984; Reid *et al.*, 2021). After the retreat of Pleistocene glaciers, the species has repeatedly and independently recolonised freshwater environments in the Northern Hemisphere from widespread marine ancestral populations (Bell & Foster, 1994). The repeated directional evolution of similar phenotypes in similar environmental conditions indicates that some adaptive traits may be under strong, consistent natural selection. A key phenotypic trait that has received particularly close study is the loss of lateral armour plate in freshwater populations (Bell *et al.*, 2004; Barrett *et al.*, 2008), as plates are believed to play an important role in defence against predators (Reimchen,

2000). Different ecomorphs can be distinguished according to the number of plates (Münzing, 1963; Hagen & Gilbertson, 1973): completely-plated, observed in marine and brackish environment and potentially anadromous; partially-plated, found in brackish environments; and low-plated, found in freshwater and brackish environments. In some locations, contact zones are found with distinct ecomorphs co-existing, such as the types found in marine and freshwater habitats and the possibility of their hybrid offspring occurring at low or high rates (McPhail, 1994; Hendry *et al.*, 2009). Population genetics suggest that rates of gene flow across these contact zones can often be surprisingly high (McPhail, 1992; Jones *et al.*, 2006b; Raeymaekers *et al.*, 2014).

A gene with a strong, well-characterised contribution to variation in degree of plating is Ectodysplasin (EDA) (Colosimo, 2005; Defaveri *et al.*, 2011; Shimada *et al.*, 2011b). Other genes known to be involved in parallel evolution in freshwater populations include: the Kit ligand, Kitlg, which affects skin and gill pigmentation (Miller *et al.*, 2010); the pituitatry homeobox 1 gene, PITX1, which affects the presence or absence of the pelvic spine (Shapiro *et al.*, 2006); and Fatty acid desaturase 2 (Fads2), which can affect fatty synthesis ability (Ishikawa *et al.*, 2019). All these examples show that the contribution of a single gene can have a large effect on the potential for stickleback to adapt to different environmental pressures when colonising new environments, and therefore, drive adaptation in these populations.

The sticklebacks of the Burrishoole catchment in the west of Ireland are an example of one such instance of parallel evolution (the process by which two or more populations living in the same environment develop similar adaptation or characteristics) in a contact zone. Previous work in Ireland (Ravinet *et al.*, 2015) and in this system in particular observed high phenotypic diversity and identified the frequently-characterised ecomorphs: a low-plated freshwater resident in Lough Feeagh; a completely-plated

anadromous ecomorph in brackish Lough Furnace; a low-plated resident ecomorph, also in Lough Furnace; and a partially-plated ecomorph also resident in Lough Furnace (Figure 3.1) (Ravinet *et al.*, 2015). These ecomorphs were found to differ in morphological traits such as body shape, pelvic and dorsal spines, and gill raker length and numbers. Both neutral and QTL-linked microsatellites markers (including two EDA-linked markers) were able to differentiate between certain pairs of ecomorphs and locations: individuals from Lough Feeagh differentiated from all of the ecomorphs in Lough Furnace (Figure 1), and the completely-plated anadromous ecomorph in Lough Furnace was strongly differentiated genetically from morphologically discriminated low plated and a partially plated ecomorphs found in Lough Furnace. However, there was little to no genetic evidence to distinguish differentiation between these low-plated and the partially-plated Lough Furnace ecomorphs. Also, surprisingly, QTL markers linked to EDA were not strongly associated with ecomorph in the system. There was inadequate evidence for hybrids between genetically distinct population pairs i.e. the completely-plated and low-plated individuals. Due to the apparent lack of hybrids, a certain degree of reproductive isolation is suspected in this system, even though the reproductive barriers remain unknown. In Chapter 2, I found only sufficient morphological evidence based on plate development to justify two distinct ecomorphs in Lough Furnace: a completely-plated ecomorph and a low-plated ecomorph. However, there was no support for the third ecomorph suggested by Ravinet *et al*. (2015), corresponding to the partially-plated ecomorph, was first reported by Münzing (1963) in various European populations. Importantly, ecomorphs overlap at the same sampling sites in Lough Furnace during the breeding season, implying an apparent absence of spatial and temporal reproductive barriers. Also in work undertaken in Chapter 2, viable hybrids between two ecomorphs were produced in a breeding experiment under semi-natural conditions. Hence, though

hybrids are potentially produced, their apparent absence (Ravinet *et al*. 2015) suggests other, unknown reproductive barriers may be operating. Understanding gene flow between these different ecomorphs and their genetic structure would highlight the dynamic in the Burrishoole system.

The aim of the study was to test for population genetic differentiation and genetic structuring among the different ecomorphs populations in the Burrishoole system, and to gain some understanding of the processes that drive this differentiation. This study builds on that of Ravinet *et al.* (2015), which used neutral and QTL-linked microsatellites and showed that there was very little evidence for gene flow between the different ecomorphs. It also extends the study of Ravinet *et al*. (2015) temporally and spatially. Temporally by comparing ecomorphs sampled in Loughs Furnace and Feeagh in 2009 and 2010 relative to new samples collected contemporaneously between 2018 and 2020; spatially by collecting samples of the different ecomorphs over a broader range of sites in Lough Furnace and including a sample collected from a nearby coastal site at Muingdoran. Here, I used targeted SNP genotyping of candidate selected and candidate neutral loci to study the spatial and temporal distribution of the ecomorphs. For candidate functional SNPs, I also assessed whether any of these loci had power to differentiate marine and freshwater populations as reported by Jones et al. (2012c) and Ferchaud et al. (2014). I hypothesize that (1) the different morphotypes identified in Lough Furnace with potential hybrids, would differentiate genetically from sampled freshwater and marine populations. I hypothesised that (2) the segregation and fixation patterns in functional and neutral loci reported previously in the published literature would also apply to stickleback populations found in the Burrishoole system and would be evidence of broader geographical processes of parallel evolution.

Materials and methods

1. Study system

The Burrishoole catchment (N53°55′22″, W9°34′20″), located in Co. Mayo in the west of Ireland, is composed of a network of rivers and lakes connected to the Atlantic Ocean via the estuary Clew Bay. The two main lakes are Lough Feeagh (410 ha) and Lough Furnace (141 ha) (Poole & de Eyto, 2006; Cassina, 2012). Lough Feeagh is an oligotrophic freshwater lake with a maximum depth of 46 m (de Eyto et al., 2016). Feeagh is connected to Furnace via two channels: the natural Salmon Leap and the artificial Mill Race.

Lough Furnace is a deep, permanently stratified, brackish tidal lagoon (Cassina, 2012; Cassina *et al.*, 2013). The lake has a strong and shallow pycnocline at 2.4 m, with high dissolved oxygen between it and the surface, and virtual anoxia under 6-7 m depth. Salinity at the surface is rather low (from 0 to 4 PSU) in the northern part of the lake with the freshwater inputs from Lough Feeagh. In the south of the lagoon is more brackish in nature with salinity values in the range of 10 to 22 PSU, increasing to full oceanic levels of salinity in Clew Bay of approximately 30 PSU. This bay has high habitat diversity and has been designated as an Area of Conservation (NPWS, 2015). From spring to summer, temperatures above the pycnocline are warm but variable (15-22°C), but below are colder and stable (~11.6°C). In winter and autumn, the pattern changes dramatically as the temperature is warmer below the pycnocline and stable (~11.6°C) while being relatively cold at the surface (3-8°C). Occasionally, the lake experiences a rapidly occurring near-total mixing events where lake temperature and dissolved oxygen can change radically within the space of a single day (Kelly *et al.*, 2018a,b). Strong winds during stormy

events can also be responsible for upwelling events in the lake, which brings up to the lake surface saline water (~22 PSU from deep in the lake). In comparison, Lough Feeagh is monomictic and purely freshwater (0 PSU). Mixing events are extremely rare (e.g., Storm Hector, 2018), and stratification rapidly re-establishes (e.g., two weeks) (Calderó-Pascual *et al.*, 2020) .

To provide a geographically proximate wholly marine stickleback outgroup a rocky shore location was sampled at Muingdoran, Co. Mayo (N54°06'10.4"N, 9°59'28.6"W). The site is composed of pools, crevices and channels. The rock pools are completely exposed to Atlantic Ocean weather conditions, and the salinity is very high (22-36 PSU). The pools are directly connected to the ocean at high tides but are usually separated during low tides. In addition, seven individuals from the West Hebrides sampled in 2007 were used in this study, captured by trawling.

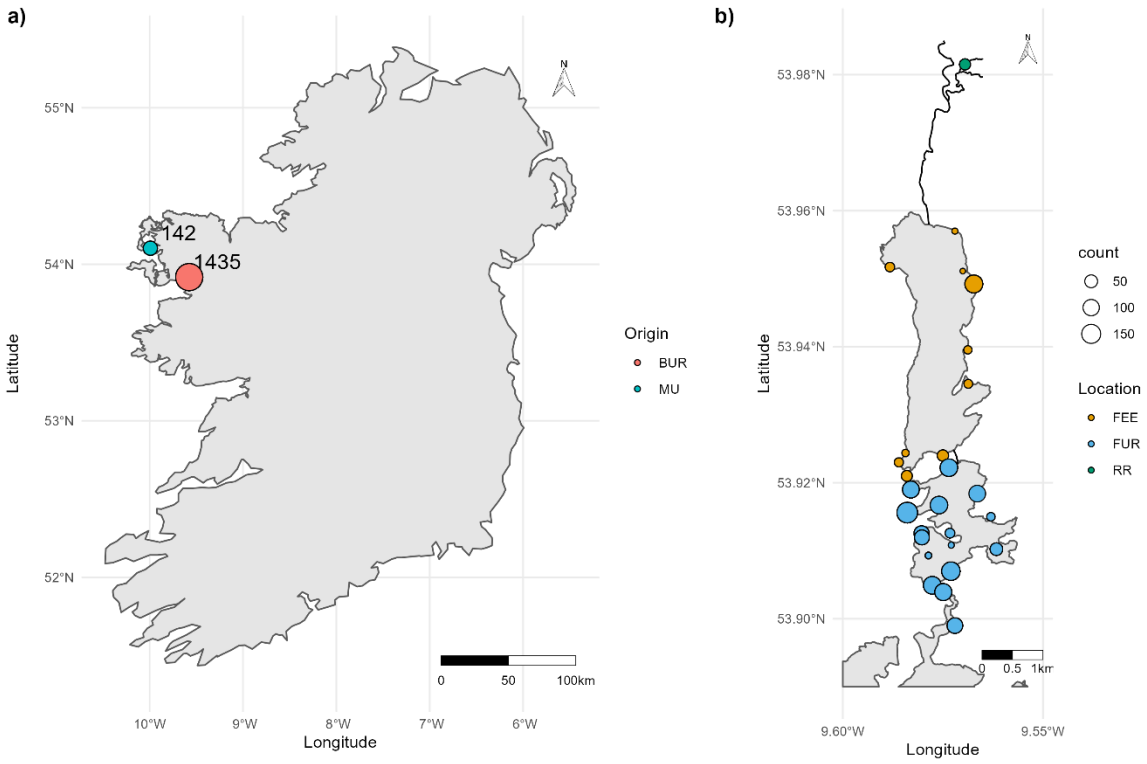


Figure 3.1. a) Map of Ireland showing the two main locations on the west coast of Ireland: Muingdoran (blue) and Burrishoole (red) with the number of fish caught per

location; b) The Burrishoole system with the three main locations: Lough Feeagh (yellow), Lough Furnace (blue) and Rough River (green). The circle size is proportional to the number of fish caught at a given site.

2. Sampling from wild populations

I assembled 1,599 samples from eight different years between 2007 and 2020 (Table 3.1). Stickleback samples from 2009 and 2010 were those of Ravinet *et al*. (2012) (n=337), and fish sampled during the SALSEA-Merge post-smolt Atlantic salmon survey (http://salmonatsea.com/the-salsea-merge-project/) carried out in 2007 as a by-catch (n=7).

Between 2017 and 2020, I collected samples from 17 different sites: four in Lough Feeagh (n=181) and from 12 different sites in Lough Furnace (n=923) extending out into the estuary of Clew Bay (Figure 3.1). Unbaited minnow traps (silver galvanized Gee-type model #1279 Frabill, Jackson, Wisconsin, USA) were set once a month during the reproductive season from april to august, and three other samplings were carried out during autumn, and at the beginning and the end of winter, to capture individuals outside the spawning season. Additionally, samples were collected at Muingdoran in Tullaghan Bay, which is an exposed rocky shore location approximately some 50 km from the mouth of the Burrishoole system during the breeding season from june to august in 2019 (n = 142).

Unbaited traps were set 1 to 3 meters from the shoreline, at 0.5 to 2.5 meter depth. Traps were deployed early in the morning and recovered 48 hours later to cover two tide cycles (with two high tides and two low tides) in Furnace and the estuary. Each time traps were collected, salinity and water temperature were recorded at the trap site using a temperature-salinometer (WTW Multi 340i Handheld Multimeters, Germany).

All fish were brought back alive to the laboratory at the Marine Institute Research Facility in Newport, Ireland, and humanely euthanized with an overdose of MS-222 solution (1.0 g/L of Tricaine methane sulfonate, FVG, Ireland).

The number of lateral plates was counted on each side of euthanized fish under a binocular magnifier scope (Stereo, Euromex, Holland). Fork length and total length were measured to the nearest 0.5 mm using callipers, and wet mass was measured to the nearest 0.1 g. A fin clip (ca. 5-7 mm^2) was taken and preserved in absolute ethanol for later genetic identification. Each fish was categorised according to their average number of plates on both sides of the body as: low-plated (LP) when the average number of plates was less than seven and completely-plated (CP) for an average number of plates was greater than 20 (see Chapter 2). Juveniles (not sexually mature) were not assigned to a plate type, as plates are not fully developed until the fish is at least five months old (Bell, 1981; Banbura, 1987). An additional seven samples collected as part of a separate study in the Hebrides in 2007 were genotyped to act as an outgroup.

Initially, 2,192 samples from the different locations were included in genotyping. Unfortunately, many of these samples were genotyped with a rate of less than 90% i.e. for the number of markers on the panel resolved successfully for that individual and these individuals were subsequently excluded (n = 564). Other samples, where provenance was unknown or uncertain (n = 29), were also excluded from analysis.

Table 3.1. Number of fish sampled per ecomorph (low-plated: LP, and completely-plated: CP), habitat (FEE: Lough Feeagh, FUR: Lough Furnace, MAR_HEB: marine fish from Hebrides, MAR_MU: marine fish from Muingdoran in Ireland, RR: Rough River) and per year.

	2007	2009	2010	2011	2017	2018	2019	2020
FEE_LP	0	28	95	0	74	72	22	13
FUR_CP	0	2	10	0	0	37	43	42
FUR_LP	0	11	169	0	69	292	390	50
MAR_HEB	7	0	0	0	0	0	0	0
MAR_MU	0	0	0	0	0	0	119	23
RR	0	0	22	1	1	3	4	0

3. SNP selection and primer design

Of 260 candidate SNPs reported for sticklebacks from the panels of Jones *et al.* (2012a) and Ferchaud *et al.* (2014), I selected 25 putatively adaptive (markers that showed previously divergence between freshwater and marine ecotypes) and 25 putatively neutral (markers that did not show divergence between ecotypes based on neutral models). SNP loci were selected, henceforth called ‘adaptive’ and ‘neutral’ markers respectively. The chosen adaptive SNPs were those with the highest reported power for discriminating marine and freshwater stickleback (S1) and the neutral SNPs were a random subsample of those markers deemed to be not associated with any adaptive trait. Sequences of these selected SNPs were BLAST searched on Three Spine Stickleback Genome Browser (https://stickleback.genetics.uga.edu/) to verify matches with target regions. Three sequences matched non-target regions of the genome, but two of these had sufficiently low identity and query scores for the risk of non-specific amplification to be minimal. The third had a higher identity and query score which could potentially lead to genotyping error and linkage disequilibrium, and was therefore excluded from subsequent analysis.

Primers were designed by LGC Biosearch Technologies based on the flanking sequences selected for this study, and can be found on NCBI with their accession number (S1). The SNP sequence, the name, and the associated genes were found on the NCBI (National Center for Biotechnology Information (NCBI), 1988) (accessed 2017 Apr 06) and are given in the supplementary material (S1).

4. DNA extraction and SNP assay method

DNA was extracted from fin clips using the Wizard SV 96 Genomic DNA Promega kit, following the manufacturer's protocol. DNA concentration was assessed for each sample (AccuBlue® High Sensitivity dsDNA Quantitation Assay) and diluted to approximately 5 ng/µL for use in amplification of SNP loci.

I used a Kompetitive Allele Specific PCR (KASP) amplification approach of previously identified candidate neutral and adaptive (locus linked to a gene function), SNPs selected from two important recent stickleback genomic studies Jones *et al.* (2012) and Ferchaud *et al.* (2014). The KASP method is based on the detection of both alleles at a specific SNP site in a single reaction. It uses two forward primers with allele-specific pattern at the targeted site and one universal reverse primer. In the first round of PCR, the two forward primers will bind to the specific DNA sequence and elongates. At the second round of PCR, the universal reverse primer will also attach to all DNA and elongate. In the last PCR round, specific primers fluorescence will be activated, allowing the SNP genotype to be identified based on competitive fluorescence rates.

All DNA samples were diluted to 5 ng/µL for use in all SNP assays. For each KASP assay, the following components are required: 5 ng of dried template DNA 3 µl of master

(Taq polymerase and fluorescence resonance energy transfer (FRET) cassettes), 0.055 µl of KASP assay mix (composed of the two competitive allele-specific forward and 2x reverse primers) and 3 µl of double-distilled H_2O.

The KASP amplifications were performed on a Hydrocycler (LGC Biosearch Technologies) platform. The initial PCR conditions started at 94°C for 15 min, followed by 10 cycles of 94°C for 20s and 65°C for 60 s. Each cycle decreases by 1°C to reach 55°C. The final PCR cycle consisted of with 31 cycles of 94°C for 20 s and 55°C for 1 min.

I used a CLARIOstar® *Plus* microplate reader (BMG Labtech, Ortenberg, Germany) to assess the fluorescence emission for each reaction. Fluorescence signal was acquired at 520 nm (green) and 556 nm (yellow) for 2 min at 25°C. All assays included negative controls (with no target DNA) and were fluorescence-free in all cases.

5. SNP genotyping and statistical analysis

SNPs were visualised with the SNPViewer tool (LGC Biosearch Technologies) and genotypes were assigned using the KlusterCaller tool in Kraken software (Wood & Salzberg, 2014).

As an alternative to blind testing subject to some methodological issues associated with the analysis of bi-allelic SNP markers), I performed Bayesian population genetic clustering analyses in STRUCTURE v 2.3.4 (Pritchard *et al.*, 2000) using ecomorph as a prior as reported in Chapter 2. I performed five independent runs for each K value (1-8) with a burn-in period of 100,000 iterations, and a total run length of 100,000 MCMCs (Markov Chain Monte Carlo) replicates, with an admixture model and correlated allele frequencies. Results from the multiple runs were assessed with the Evanno approach

(Evanno *et al.*, 2005) and using the maximal value of L(K) returned by Structure (Zeisset & Beebee, 2001) or where the variance between runs of successive values of K increased substantially following a maximal peak. I used the function *evannoMethodStructure()* from the Pophelper package v 2.3.1 (Francis, 2017) and STRUCTURE harvester (Earl & vonHoldt, 2012) to identify the optimum number of genetic clusters. Partitioning plots among individuals to the different clusters were generated with this same package. Relationship among genetic clusters/groups of individuals were visualised using Neighbour-joining dendrogram constructed in MEGA (Tamura et al., 2007) using Nei's D_A genetic distance (Nei *et al.*, 1983). Bootstrap (1000 replications) values for tree branches were calculated using POPTREE (Takezaki et al., 2010). In constructing neighbour-joining dendrograms, I restricted the data individuals with a maximum assignment to a cluster of > 0.8.

To test for spatial genetic structure within the higher-level sampling sites (Rough River, Feeagh, Furnace, and marine sample from Muingdoran), I undertook a hierarchical-like analysis by running STUCTURE for sub-sets of data organised by ecomorph group (Chapter 2) and identifying the minimum K within each ecomorph. I undertook the same approach for the temporal aspect by again sub-sampling on the basis of ecomorph and comparing the data between two temporal periods: 2009-2010 samples from Ravinet *et al.* (2015) versus samples collected in this project between 2017-2020 within each ecomorph.

Discriminant Analysis of Principal Components (DAPC) and statistical analyses were performed as outlined below in the statistical software R v 4.1.0 (R Core Team, 2018), and all figures were produced using the ggplot2 package v3.3.5 (Wickham, 2016).

Blind population genetic clustering analyses were performed on all markers together, then on different sets of markers separately (all neutral, all adaptive, eight adaptive and finally two adaptive). I first performed PCA-based clustering using the *find.clusters()* function from the adegenet package v 2.1.5 (Jombart, 2008). I repeated the analysis ten times over a K range of 1-15, collected the BIC values from each repeated analysis, and inspected them visually to identify candidate best K values. This was followed up with two DAPC analyses using adegenet's *dapc()* function: one based on the clustering suggested by *find.clusters*(), and one based on the *a priori* biological groups from our database (location and ecomorph).

Standard population genetic statistics (allele frequencies, H_O, H_E, Hardy-Weinberg equilibrium [HWE], F_{ST}) were performed using functions *fastDivPart()* and *divBasic()* from the DiveRsity package (Keenan *et al.*, 2013).

To test for signatures of selection, I used the Bayesian likelihood method implemented via reversible jump MCMC in the BayeScan software (Foll & Gaggiotti, 2008). BayeScan estimates the probability that a locus is under natural selection by calculating a Bayesian factor (ratio of the posterior probabilities of two models) of neutrality v. selection. Log10(Bayes Factor) values > 0.5 are typically considered under substantial evidence of selection in these analyses, while Log10(Bayes Factor) estimates between 0 and 0.5 are considered to be under weak selection. I used 20 pilot runs of 5,000 iterations, a burn-in of 50,000 iterations, a thinning of 10 with a resulting total number of 100,000 iterations and a prior odds ratio of 10.

To identify full-sibs among samples, where it was deemed necessary because of occurrence of two or more groups within the same ecomorph sample (in the marine individuals from Muingdoran) and also because it was felt that in sampling individual

rock pools there was a high possibility of collecting multiple individuals from the same family, I used COLONY software v2.0.5.0 (Jones & Wang, 2010).

Phenotypic groups are henceforth referred to by the following abbreviations: Rough River (or Srahrevagh River) = RR; low-plated from Lough Feeagh = FEE_LP; low-plated from Lough Furnace = FUR_LP; completely-plated from Lough Furnace = FUR_CP; Irish marine group sampled at Muingdoran = MAR_MU; and Hebrides marine = MAR_HEB.

Results

1. Genotyping success and genetic diversity in the Burrishoole:

Among the 50 SNP loci tested, 19 failed to produce reliable fluorescence-allele spreads such that genotypes could not be reliably scored. These loci were not considered further. Of the remainder, three primer-set sequences appeared to match non-target regions of the genome although two of these had sufficiently low identity/query scores for the risk of non-specific amplification to be minimal. A third locus primer-set had a higher identity/query score to non-specific regions of DNA, which could potentially lead to genotyping error and linkage disequilibrium, therefore, this locus was also excluded from further analysis. This left 32 loci for analysis, 22 of which were considered to be neutral and 10 adaptive based on the published literature. Initial testing of genotypes at these loci was undertaken among subsets of individuals (segregated by ecomorph/location) in order to assess success in terms of the proportion of individuals scored, level of heterozygosity and degree of linkage disequilibrium. One locus (from the neutral set) was observed to have substantial and highly significant deficit of heterozygotes among all sample subsets for both location and ecomorph. As this marker was considered to be unreliable it was

not used subsequently. Among all the remaining loci (31), two loci showed evidence for significant and similar linkage associations (chrI_21689292 and chrI_21663978) not unexpectedly as these loci were located quite closely to each other on the same chromosome (chromosome I). In order to avoid pseudo-replication, only one of these was included subsequently in the analysis, in this instance the chrI_21663978), which was the marker that was scored most successfully among individuals.

Genotyping success was high for all loci in the chosen panel across the 1,599 samples included in downstream analysis, and only one SNP out of the 30 had a genotyping success under 90% (88.4%, Table 3.2). Twenty-seven loci were successfully genotyped at over 98%. Eight loci had a major allele frequency less than 90% and only six had a major allele frequency under 70%. Out of the 30 loci selected, 23 had a HWE p-value (P) under 0.05, indicating significant departure from HWE. At this stage in the analyses, I was not worried by HWE departures, as they may result from population genetic structure within the pooled dataset. All 1,599 individuals were used for downstream analyses (Table 3.2).

Table 3.2. Summary of the different markers information: marker ID, the two alleles with the major first, the marker type (presumed neutral vs presumed selected based on published literature), the major allele frequency in all dataset, the p-value (P) testing the Hardy-Weinberg Equilibrium (HWE) on all the individuals and the percentage of unsuccessful genotype per locus. The markers in bold are the one with genotyping success <90%.

Marker	**Alleles**	**Type**	**Maj.Al.Freq**	**HWE p-value**	**Unsuccess (%)**
21858	G/A	neutral	97.8	0.001	0
22319	C/T	neutral	59.4	<0.001	0.5
31954	T/A	neutral	70.2	<0.001	11.6
11120	G/A	neutral	77.6	<0.001	0.8
13177	C/T	neutral	79.9	<0.001	0.3
20574	G/A	neutral	59.6	<0.001	0.5
27027	A/G	neutral	58.2	<0.001	4.3
16548	C/A	neutral	77.3	<0.001	0
28703	C/A	neutral	70.3	0.590	0.3
4390	C/T	neutral	88.7	<0.001	0.3
35752	G/A	neutral	96.9	0.185	0.3
5812b	A/G	neutral	96.0	0.003	0.9
20825	G/A	neutral	94.7	<0.001	0.8
32977	A/C	neutral	67.1	<0.001	0.4
7275	C/T	neutral	74.5	<0.001	1.1
10561	G/A	neutral	97.1	<0.001	3.8
1139	C/T	neutral	70.2	<0.001	0.4
1800	G/T	neutral	68.4	0.384	0.7
21643	C/G	neutral	71.9	0.804	1.2
9206	G/A	neutral	91.4	0.054	0.1
33523	C/A	adaptive	57.8	0.101	0.1
20238	G/A	adaptive	96.0	1.000	0.1
6844	T/A	adaptive	87.4	<0.001	0.8
2113	T/G	adaptive	89.3	0.024	0.6
5200	C/T	adaptive	83.9	<0.001	0.8
chrXXI_5793103	G/A	adaptive	94.2	0.018	0.9
chrXI_5715882	A/G	adaptive	81.9	<0.001	0.2
chrI_21663978	G/A	adaptive	68.4	<0.001	1.6
chrI_21951727	A/G	adaptive	86.6	<0.001	0.7
chrXIV_11360680	T/C	adaptive	83.0	<0.001	0.4

2. Population genetic structure all markers:

STRUCTURE and DAPC – All markers

STRUCTURE analysis using all markers and carried out on all individuals, suggested a best number of putative ecomorphs of 3: a group with RR and FEE_LP individuals, another group with FUR_LP and one consisting of FUR_CP, MAR_MU and MAR_HEB. Some individuals were mismatched for phenotype and genotype, and details in respect of potential explanations are outlined in S3.5. Neighbour-joining dendrograms with strong bootstrap support located freshwater populations (RR and FEE_LP) close together on branches of the dendrogram. Similarly, completely-plated individuals (FUR_CP and MAR_MU) were found close together. The FUR_LP group was located in a position intermediate to the freshwater and completely-plated branches (S3.6). All details and analysis with STRUCTURE using all markers are detailed in S3.2.

DAPC analysis presented a similar pattern as to the STRUCTURE analysis, with a suitable best K value suggesting between 1 and 4 groups (S3.4). On the basis of K = 4 and using fish phenotype as a prior, the first axis discriminated individuals based on their number of lateral plates and location, with all completely-plated (FUR_CP and MAR_MU) grouping closely together, low-plated (FEE_LP and RR) grouping together, and the FUR_LP being intermediate between these two main groups (Figure 3.8; S3.7). All the details and analysis using a DAPC approach are detailed in S3.7.

When comparing samples across two periods of time (2009-2010 and 2017-2020), there was low temporal instability within each specific location, all details of this analysis can be found in S3.10.

No single locus showed consistent patterns of deficit or excess of heterozygotes across populations, except for locus chrI_21663978 (linked to salinity tolerance), which presented a deficit in heterozygotes for both Rough River group (H_O = 0.23 and H_E = 0.43) and the phenotypic FUR_LP fish from the Lough Feeagh (freshwater) genotype (H_O = 0.38 and H_E = 0.49) (S3.13). The lowest F_{ST} value (all markers) was observed between RR and FEE_LP (0.062); similarly, MAR_MU and MAR_HEB (0.054) (S3.13). The highest values observed were between FUR_CP and RR-FEE_LP (0.47 and 0.41 respectively), followed by MAR_MU and MAR_HEB compared to RR and FEE_LP (ranging from 0.29 to 0.39). F_{ST} values and all summary statistics are explained in S3.14.

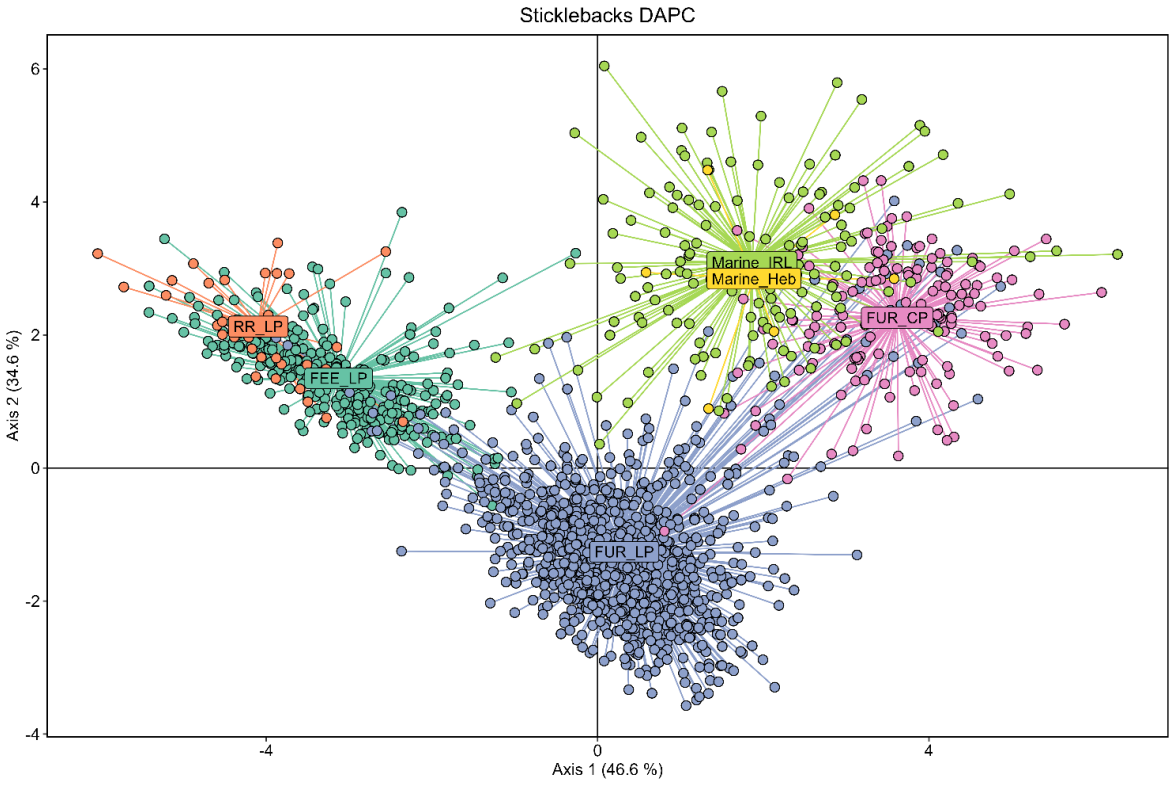


Figure 3.2. Scatter plots of all samples using the two principal components of DAPC analysis using ecomorph as a prior with K = 4. Individuals are represented as dots and the groups as ellipses. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

3. Comparison between neutral and adaptive markers

a) General pattern of neutral and adaptive SNPs

In these analyses, some groups were excluded for the following reasons: unassigned individuals not belonging to a specific genetic cluster and FW fish in Lough Furnace of ambiguous origin leaving only the major groups (RR, FEE_LP, FUR_LP, FUR_CP, MAR_MU, MAR_HEB). The global F_{ST} for both neutral and adaptive markers was 0.23. F_{ST} was estimated marker by marker in each group and found, by visual inspection, that adaptive markers did not show any specific pattern compared to the neutral markers, except for two: chrI_21951727 (F_{ST} = 0.84, (physically linked to two genes linked to salinity tolerance) and chrXIV_11360680 (F_{ST} = 0.68, linked to metabolism). One previously presumed neutral marker (27027) also showed a higher F_{ST} compared to the other (F_{ST} = 0.57).

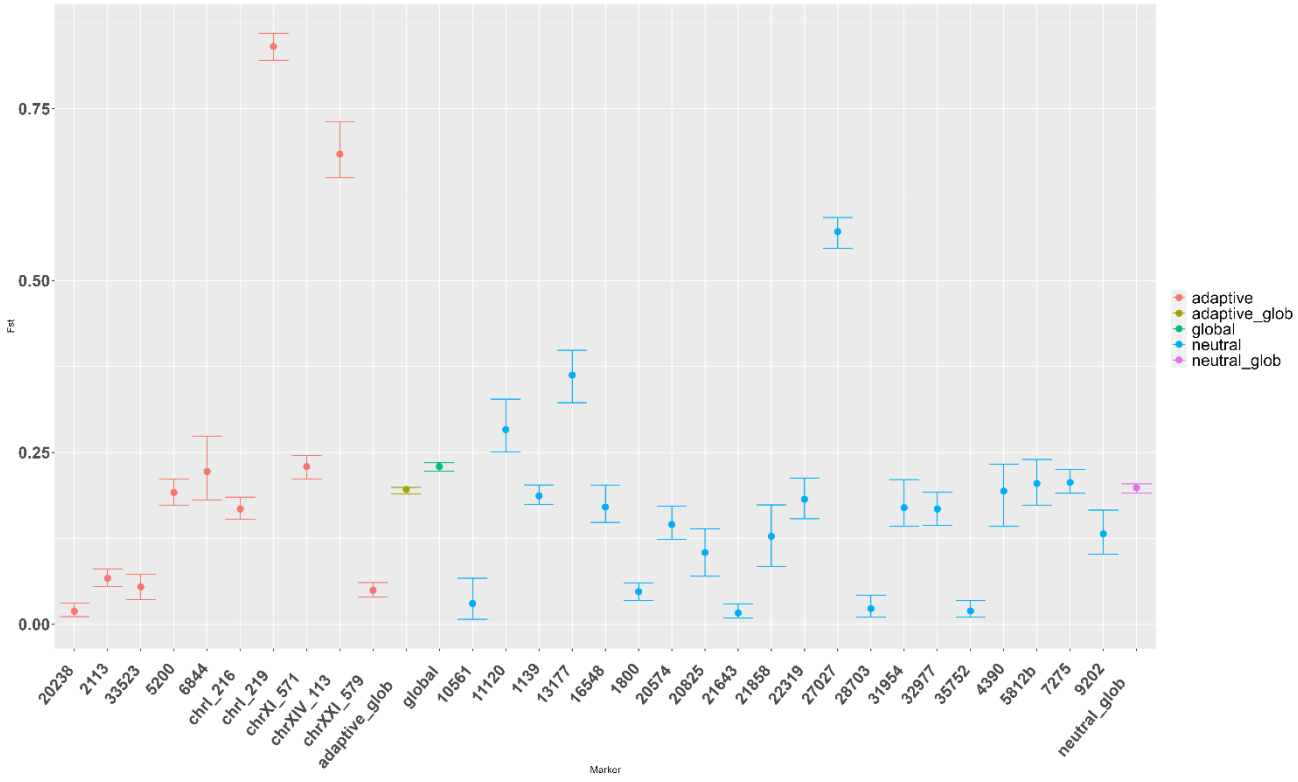


Figure 3.3. F_{ST} value for each locus (adaptive, neutral, neutral global, adaptive global and global) calculated on the previous groups.

When comparing allele frequency variability among these four groups between neutral and adaptive makers, fish from FEE_LP had the highest number of monomorphic or very low variability (<0.05) loci (53.3% overall) (S3.13). Most of the adaptive loci were monomorphic or with low variability with 66.7% compared to the neutral with 47.6% for the FEE_LP. Completely-plated fish are the second group with the highest proportion of low variability loci (46.7%). However, the proportion of low variability loci was highest for the neutrals, with 88.9% compared to the adaptive with 28.6%. Irish marine individuals (MAR_MU) had an overall proportion of low variability loci of 20%, but the differences between neutral loci (4.8%) compared to the adaptive loci (55.6%) was important. In FUR_LP, the loci variability was not different between neutral and adaptive (28.6% and 33.3% respectively).

I ran STRUCTURE on these four groups with neutral markers only (Figure 3.4). The best number of clusters according to the Evanno method was K = 2, with one cluster containing mainly RR and FEE_LP, and the other cluster comprising all other groups (FUR_LP, FUR_CP and MAR_MU). When using the adaptive markers only, the best number of K according to the Evanno method was K = 2, but with one cluster containing fish from RR, FEE_LP and FUR_LP, and the other containing FUR_CP, MAR_MU and MAR_HEB.

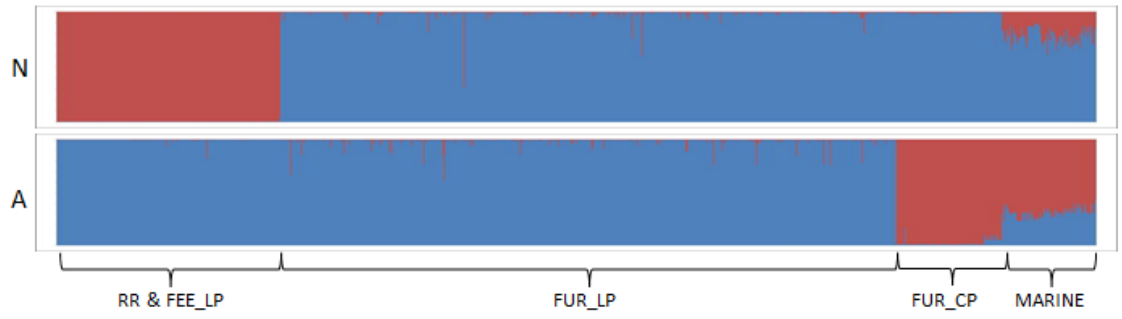


Figure 3.4. The four major ecomorphs with their STRUCTURE assignment probability to belong in a cluster, grouped visually by ecomorph for K = 2: with only neutral markers (top) and with only adaptive markers (bottom).

b) Neutral markers only

When comparing F_{ST} values between group pairs with all the neutral marker, the highest levels were found between the freshwater groups (RR, FEE_LP) and the brackish-marine groups (FUR_LP, FUR_CP, MAR_MU, MAR_HEB) with values ranging from 0.19 to 0.33 (Table 3.3). The lowest F_{ST} values were found between RR and FEE_LP (0.04). The F_{ST} compared among the various brackish-marine groups had values ranging from 0.04 to 0.18.

Table 3.3. Summary of FST value for all neutral markers: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish marine fish, MAR_HEB = marine fish from the Hebrides.

	RR	**FEE_LP**	**FUR_LP**	**FUR_CP**	**MAR_MU**	**MAR_HEB**
RR	0.00					
FEE_LP	0.04	0.00				
FUR_LP	0.25	0.20	0.00			
FUR_CP	0.33	0.32	0.15	0.00		
MAR_MU	0.23	0.24	0.14	0.16	0.00	
MAR_HEB	0.30	0.22	0.09	0.18	0.04	0.00

The results of a DAPC analysis on the basis of neutral markers only, blindly first (S6) and with the phenotype as a prior (Figure 3.5) showed that the first axis differentiated freshwater samples (RR, FEE_LP) from brackish (FUR_LP, FUR_CP) and marine samples (MAR_MU, MAR_HEB) (Figure 3.5), explaining 56% of the variance. The marine samples (MAR_MU and HE) were genetically more similar to the brackish samples (FUR_LP and FUR_CP) than to the freshwater samples (RR and FEE_LP). The second axis differentiated low-plated samples (RR, FEE_LP, FUR_LP) and completely-plated samples FUR_CP, MAR_MU, MAR_HEB), explaining 28.1% of the variance.

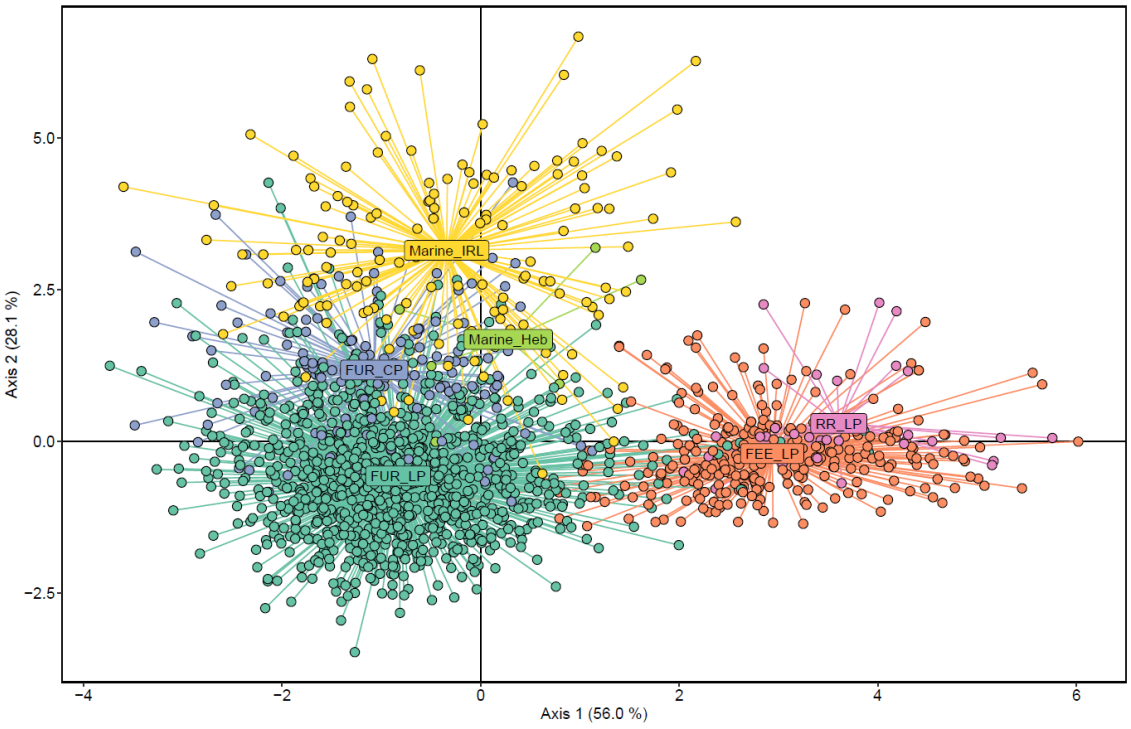


Figure 3.5. Scatter plots of all samples with only neutral markers using the two principal components of DAPC analysis using ecomorph as a prior with K = 4. Individuals are represented as dots and the groups as ellipses. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

c) All adaptive markers

When comparing F_{ST} between group pairs with all the adaptive markers taken together, the highest F_{ST} values were found between the low-plated groups (RR, FEE_LP, FUR_LP) and the completely-plated groups (FUR_LP, FUR_CP, MAR_MU, MAR_HEB) with values ranging from 0.23 to 0.71 (Table 3.4). The lowest F_{ST} values were among low-plated groups ranging from 0.10 to 0.22 and among the completely-plated groups ranging from 0.10 to 0.35.

Table 3.4. Summary of F_{ST} value for all adaptive markers: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish marine fish, MAR_HEB = marine fish from the Hebrides.

	RR	**FEE_LP**	**FUR_LP**	**FUR_CP**	**MAR_MU**	**HEB**
RR	0					
FEE_LP	0.12	0				
FUR_LP	0.23	0.10	0			
FUR_CP	0.71	0.57	0.41	0		
MAR_MU	0.49	0.36	0.23	0.35	0	
MAR_HEB	0.54	0.39	0.25	0.11	0.10	0

In a DAPC analysis using only adaptive markers, blindly first (S7) and with the phenotype as a prior (Figure 3.6) the first axis differentiated the low-plated samples (RR, FEE_LP, FUR_LP) from completely-plated individuals (MAR_MU, MAR_HEB, FUR_CP) regardless of the geographical location of the sample (Figure 3.6), explaining 64.8% of the variance. The low-plated groups (RR, FEE_LP, FUR_LP) were genetically more similar to each other compared to the completely-plated that were substantially differentiated from the low plated groups and from each other. The second axis differentiated individuals based on location with 20.5% of the variance explained, with freshwater samples (RR, FEE_LP) differentiating somewhat from brackish-marine samples including the low plated Furnace group (FUR_LP). There was no clear distinction among the various brackish and marine groups in respect of location.

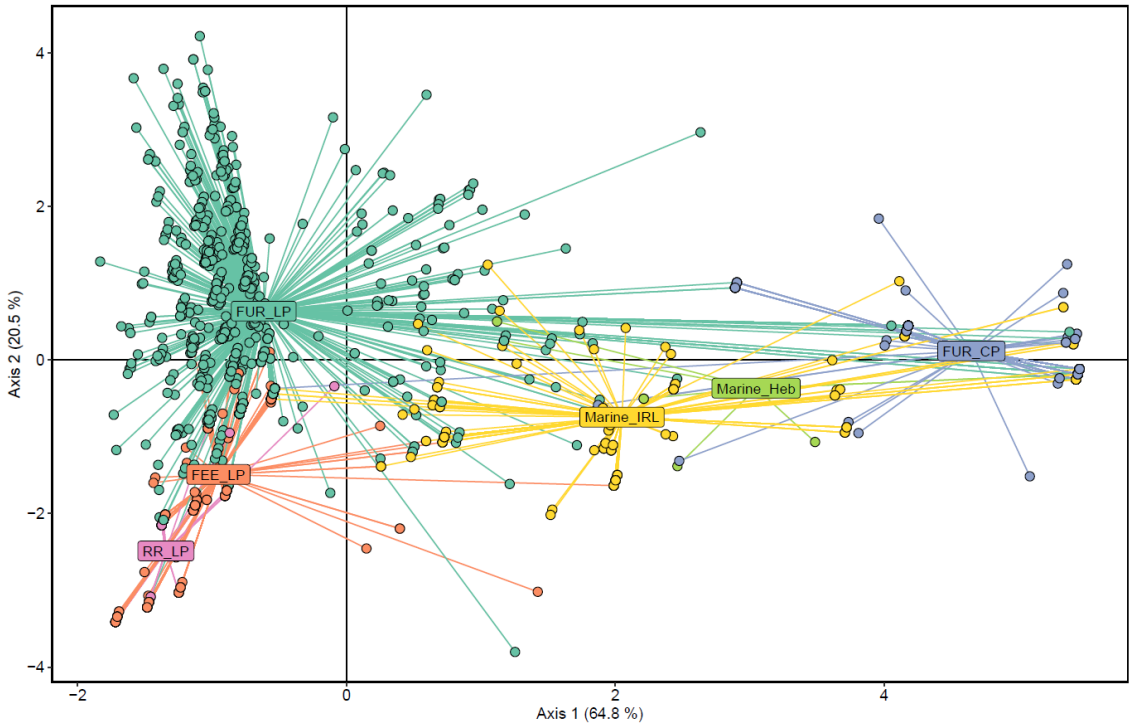


Figure 3.6. Scatter plots of all samples with only adaptive markers using the two principal components of DAPC analysis using ecomorph as a prior with K = 4. Individuals are represented as dots and the groups as ellipses. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

d) Eight adaptive markers

When comparing F_{ST} between group pairs based on the eight adaptive markers without the two strongly adaptive markers identified in the global analysis above (Figure 3.7) showed similar patterns as neutral markers, the highest F_{ST} values were between RR and all the completely-plated groups (FUR_CP, MAR_MU, MAR_HEB) ranging from 0.39 to 0.62. The low plated ecomorphs (RR, FEE_LP, FUR_LP) were very similar in respect of F_{ST} (ranging from 0.00 to 0.02) (Table 5). The lowest F_{ST} values were between the MAR_HEB and all brackish-marine groups regardless of the number of plates (FUR_LP, FUR_CP, MAR_MU) ranging from 0.02 to 0.08). All other F_{ST} values between pairwise comparisons were intermediate.

Table 5. Summary of FST value for eight adaptive markers: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish marine fish, MAR_HEB = marine fish from the Hebrides.

	RR	**FEE_LP**	**FUR_LP**	**FUR_CP**	**MAR_MU**	**MAR_HEB**
RR	0.00					
FEE_LP	0.12	0.00				
FUR_LP	0.24	0.11	0.00			
FUR_CP	0.62	0.24	0.14	0.00		
MAR_MU	0.49	0.20	0.10	0.16	0.00	
MAR_HEB	0.40	0.10	0.06	0.08	0.02	0.00

The first axis of a DAPC plotted using the eight putatively adaptive markers, blindly first (S8) and with the phenotype as a prior (Figure 3.7) differentiated individuals sampled in freshwater (RR, FEE_LP) from those individuals sampled in the brackish and marine environments (FUR_LP, FUR_CP, MAR_MU, MAR_HEB), with 61.8% of variance explained. The second axis differentiated individuals based on the number of lateral plates with the low-plated ecomorphs grouping together (RR, FEE_LP, FUR_LP), and the completely-plated clustering together (FUR_CP, MAR_MU, MAR_HEB), with the 32.9% of the variance explained.

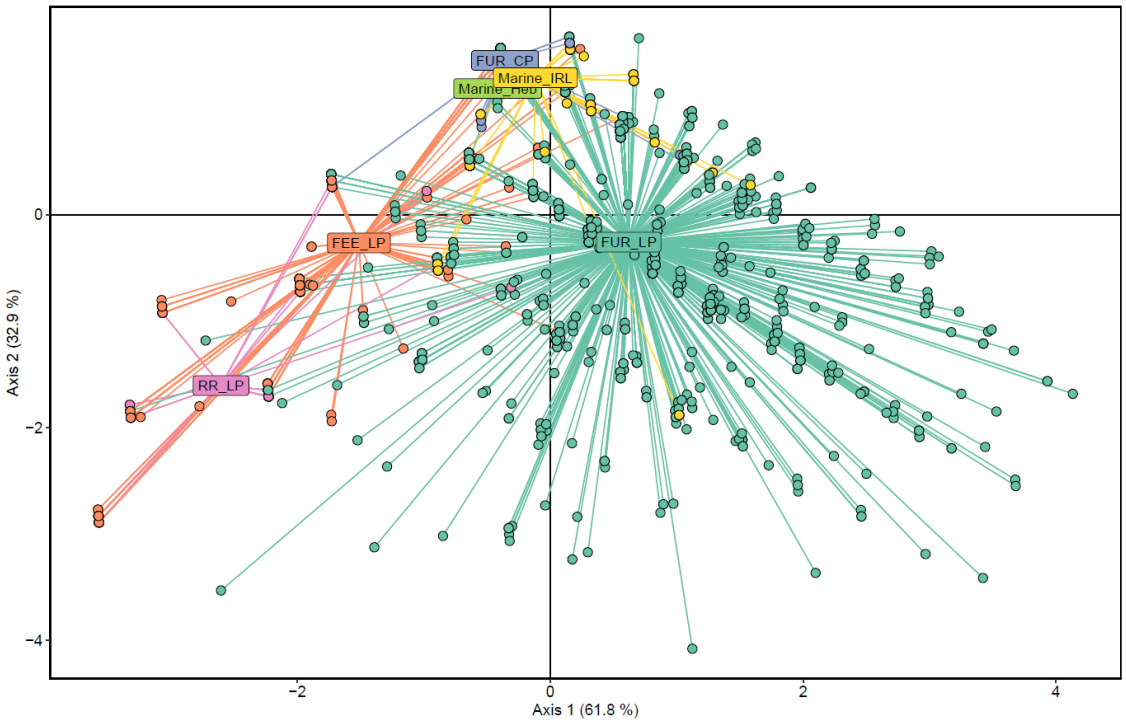


Figure 3.7. Scatter plots of all samples with only eight adaptive markers using the two principal components of DAPC analysis using ecomorph as a prior with K = 4. Individuals are represented as dots and the groups as ellipses. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

e) Two adaptive markers

Here I used the two adaptive markers that were showing differentiation pattern compared to the other: chrI_21951727 (located between two genes linked to salinity tolerance) and chrXIV_11360680 (associated with metabolic process). F_{ST}'s between group pairs compared using two markers showed a distinct pattern relative to the other adaptive markers, with low F_{ST} values found between all the low-plated groups (RR, FEE_LP, FUR_LP) ranging from 0.00 to 0.02 (Table 6). The highest F_{ST} values based on the sub-set of two adaptive markers were found among all the low and completely groups (ranging from 0.50 to 0.93). The FUR_CP group was differentiated in respect to F_{ST} to a much greater extent from the MAR_MU (0.49) group than from the MAR_HEB group (0.15). MAR_MU and MAR_HEB were also more different to each other than the low-plated groups (0.19).

Table 6. Summary of FST value for two adaptive markers: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish marine fish, MAR_HEB = marine fish from the Hebrides.

	RR_LP	**FEE_LP**	**FUR_LP**	**FUR_CP**	**MAR_MU**	**MAR_HEB**
RR_LP	0.00					
FEE_LP	0.00	0.00				
FUR_LP	0.02	0.02	0.00			
FUR_CP	0.81	0.90	0.85	0.00		
MAR_MU	0.50	0.68	0.65	0.49	0.00	
MAR_HEB	0.84	0.93	0.78	0.15	0.19	0.00

Here, I used the two putatively adaptive markers left for the DAPC analysis, blindly first (S9) and with the phenotype as a prior (Figure 3.8). The first axis strongly differentiated individuals based on their number of lateral plates, with low-plated fish all clustering together (RR, FEE_LP, FUR_LP) and completely-plated individuals (FUR_CP, MAR_MU, MAR_HEB), with 84.2% of variance explained. The second axis does not present a specific pattern of differentiation among individuals, with the 15.8% of the variance explained.

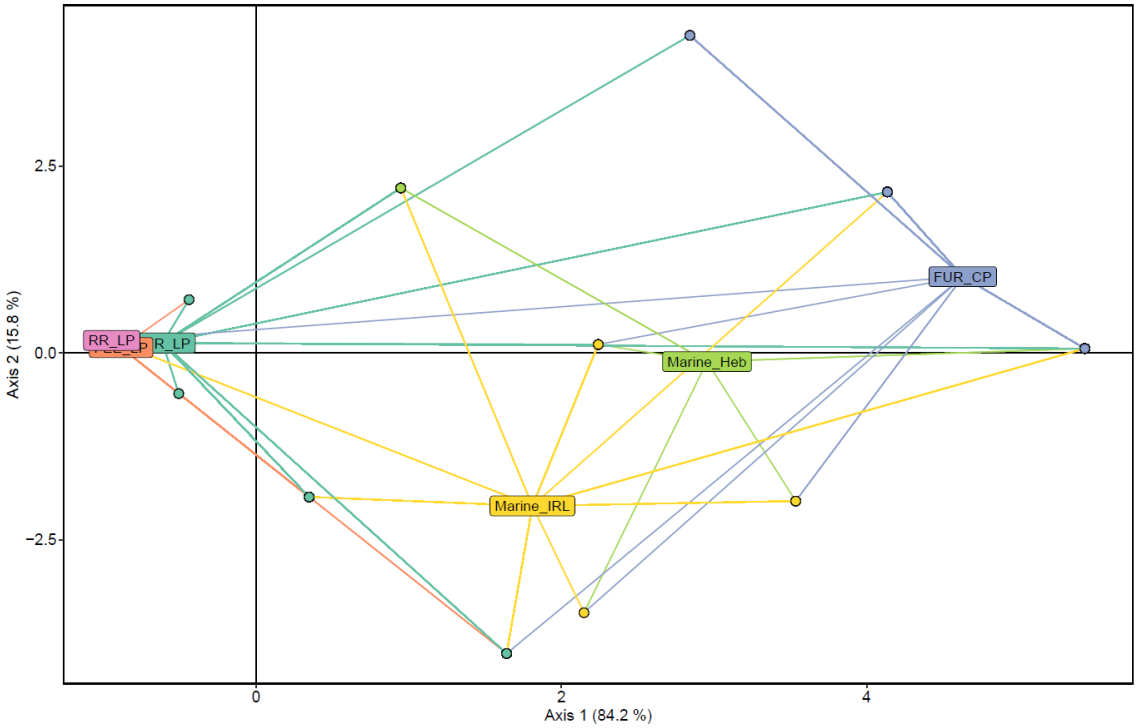


Figure 3.8. Scatter plots of all samples with the two adaptive markers using the two principal components of DAPC analysis using ecomorph as a prior with K = 4. Individuals are represented as dots and the groups as ellipses. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

f) Individual adaptive markers

When comparing pairwise F_{ST} for the marker chrI_21951727 (located between two genes linked to salinity tolerance), the lowest values were found between the low-plated groups (RR, FEE_LP and FUR_LP), ranging from 0 between RR and FEE_LP to 0.01 between FUR_LP and FEE_LP (Table 7). The highest values were observed between all the low-plated groups (RR, FEE_LP, FUR_LP) relative to the completely-plated groups (FUR_CP, MAR_MU, MAR_HEB), with values ranging from 0.14 to 0.99. The different completely-plated groups were also substantially different from each other with values ranging from 0.33 (MAR_MU and MAR_HEB) to 0.75.

Table 7. Summary of FST value for one adaptive marker chrI_21951727: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish marine fish, MAR_HEB = marine fish from the Hebrides.

	RR	**FEE_LP**	**FUR_LP**	**FUR_CP**	**MAR_MU**	**MAR_HEB**
RR	0					
FEE_LP	0	0				
FUR_LP	0.00	0.01	0.00			
FUR_CP	0.98	0.99	0.96	0.00		
MAR_MU	0.14	0.31	0.33	0.76	0.00	
MAR_HEB	0.85	0.98	0.90	0.71	0.34	0.00

When comparing pairwise F_{ST} for the marker chrXIV_11360680 (associated with metabolic process), no difference was observed between the different completely-plated groups (FUR_CP, MAR_MU, MAR_HEB) (Table 8). The values between low-plated groups (RR, FEE_LP and FUR_LP) were also very low ranging from 0.001 between FEE_LP and FUR_LP to 0.0073 between FUR_LP and RR (Table 8). The highest values were found between all the low-plated groups (RR, FEE_LP, FUR_LP) compared to the completely-plated groups (FUR_CP, MAR_MU, MAR_HEB), with values ranging from 0.63 to 0.84.

Table 8. Summary of FST value for one adaptive marker chrXIV_11360680: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish marine fish, MAR_HEB = marine fish from the Hebrides.

	RR	FEE_LP	FUR_LP	FUR_CP	MAR_MU	MAR_HEB
RR	0.00					
FEE_LP	0.00	0.00				
FUR_LP	0.01	0.00	0.00			
FUR_CP	0.64	0.78	0.82	0.00		
MAR_MU	0.63	0.77	0.82	0.00	0.00	
MAR_HEB	0.82	0.87	0.84	0.00	0.00	0.00

For the marker 27027 (presumed neutral), there was very little difference in the F_{ST} values between the freshwater groups RR and FEE_LP (0.04). No difference was found between the two marine samples, MAR_MU and MAR_HEB (0.00) (Table 9). The F_{ST} values among all the completely-plated groups (FUR_CP, MAR_MU and MAR_HEB) were small ranging from 0.08 to 0.19. High F_{ST} differences were found between the marine groups (MAR_MU and MAR_HEB) and the freshwater groups (RR and FEE_LP) ranging from 0.44 to 0.81. High F_{ST} values were observed between the FUR_LP and the two freshwater groups RR and FEE_LP with 0.73 and 0.70 respectively. The F_{ST} values observed between the FUR_CP group and freshwater groups were low, RR and FEE_LP (0.16 and 0.10 respectively). The F_{ST} values detected between the freshwater samples and the FUR_LP samples were exceptional among all other F_{ST} comparisons for these two groups relative to the comparisons for neutral, adaptive and selected adapted markers. In contrast, remarkably, $F_{ST's}$ were considerably lower at 27027 between the freshwater samples and FUR_CP sample in respect of all other comparisons.

Table 9. Summary of FST value for one neutral marker 27027: the phenotypic groups are the following: RR_LP = Rough River, FEE_LP = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_MU = Irish (Muingdoran) marine fish, MAR_HEB = marine fish from the Hebrides.

	RR	FEE_LP	FUR_LP	FUR_CP	MAR_MU	MAR_HEB
RR	0.00					
FEE_LP	0.04	0.00				
FUR_LP	0.73	0.70	0.00			
FUR_CP	0.16	0.10	0.55	0.00		
MAR_MU	0.44	0.47	0.20	0.19	0.00	
MAR_HEB	0.81	0.49	0.26	0.08	0.00	0.00

g) Evidence for selection

After running BayesScan on each of the four groups separately and all markers, only chrI_21951727 (located between two genes found previously to be associated to salinity tolerance) showed a value indicative of substantial selection (log10(BF) = 0.71). F_{ST} for this locus was 0.52 calculated within BayesScan, was twice that of other loci (mean ~0.25, ranging from 0.25-0.26). From all pairwise comparisons between each group, a single pairwise comparison between low-plated and completely-plated from Lough Furnace showed this same locus to be under weak selection (log10(BF) = 0.15). No other loci showed evidence for selection. For this locus, the F_{ST} between these two group was 0.42 whereas it was around 0.24 for all other loci. No other locus for the other pairwise comparison seem to present evidence for selection.

Discussion

Deploying a bespoke set of SNPs identified from the literature, I found significant intraspecific genetic variation in the three-spined stickleback living in sympatry and parapatry in the Burrishoole system on the west coast of Ireland suggesting four distinct populations in the system. I identified genetic clusters that corresponded with phenotypic

and spatial variation: (i) a low plated ecomorph found in the purely freshwater river and lake environments of the Rough River and Lough Feeagh respectively; (ii) based on plate number differences two distinct ecomorphs were found overlapping in Lough Furnace, a low-plated ecomorph and a completely-plated ecomorph; (iii) a completely plated ecomorph found in the geographically proximate fully marine rock pools at Muingdoran and in the open ocean off the Scottish Hebrides archipelago. Some low-plated fish that were similar genetically to those found in the Rough River and in Lough Feeagh were also found in Lough Furnace and hence were most likely to be individuals originating from the freshwater portion of the system. I found little evidence for hybridisation between the low- and completely-plated ecomorphs in Lough Furnace, leaving an open question as to what factors that might drive selection against hybridisation and/or survival of hybrids in Lough Furnace. I found population genetic structure within ecomorphs to be temporally stable and observed a degree of spatial structure within Rough River and Lough Feeagh population (consistent across the different sampling sites) and Lough Furnace, with the two different ecomorphs low-plated and completely-plated being found present at similar sites across the lake.

The presumed neutral SNPs identified from the literature primarily differentiated freshwater from brackish/marine groups, with the marine samples being genetically more similar to the brackish samples than the freshwater samples, suggesting a scenario of isolation-by-environment rather than isolation-by-distance**.** The putatively adaptive markers, in contrast, primarily differentiated LP from CP individuals, with a second minor axis of variation differentiating individuals more by the salinity of the environment from which they were collected. When analysing loci separately, however, it became apparent that the overall patterns for the 10 putatively adaptive markers were driven by two SNPs in particular (associated with salinity tolerance (chrI_21951727) and

metabolism (chrXIV_11360680) genes; though there was also some indication that the previously considered neutral SNP 27027 (chrII_13068160) could also differentiate LP and CP in Furnace. Moreover, 27027 discriminated between the low-plated freshwater samples and the low-plated brackish individuals, but interestingly not between the freshwater samples from Feeagh and the Rough River and the Furnace complete plated fish. Therefore, the 27027 marker does not seem to be associated with, or consistently differentiate fish found in Lough Furnace or Lough Feeagh or between completely-plated and low-plated individuals. It maybe that the Furnace low plated fish differ from the freshwater fish in some other unknown phenotypic aspect of their biology. The other eight adaptive SNPs (found to be associated with a range of other functions in other studies) primarily differentiated samples based on salinity environment and thus the patterns were more similar to those exhibited by the putatively neutral loci. Taken together, these findings imply a limited amount of parallelism at the SNP level with respect to morphotypes (given that only 2 of 10 putatively adaptive SNPs clearly differentiated LP from CP individuals), and that isolation-by-adaptation with respect to salinity environment might indirectly shape patterns of neutral divergence. Also these findings amply demonstrate the value in combining both neutral and non-neutral genetic variation to explain the processes that generate phenotypic variation in stickleback generally and here specifically in the Burrishoole system.

These genetically supported ecomorphs are in agreement with those previously identified by Ravinet *et al*. (2015) using 14 microsatellites and those identified in Chapter 2 based on morphological and ecological traits. A clustering analysis based on meristic and morphological data following the same method as Ravinet *et al*. (2015), identified four groups corresponding to two major phenotypic clusters and spatial location: a low-plated, small-bodied group residing in Lough Feeagh; a low-plated, small-bodied group residing

in Lough Furnace; a completely-plated, large- and deep–bodied, potentially anadromous (based on high abundance during the breeding season and virtual absence during winter; Chapter 2) in Lough Furnace; and the purely marine from Muingdoran. These ecomorphs (except for the purely marine one) were also described in Ravinet *et al*. (2015) based on morphological and genetic data. Ravinet *et al.* (2015) found these ecomorphs to be different in body shape, antipredator traits (e.g., number of lateral plate, length of spines), diet, and traits associated to diet i.e. the length of gill rakers (Ravinet, 2012). An additional 'partially-plated' ecomorph was identified by Ravinet (2012) based on morphology, though with limited genetic differentiation from low-plated fish from Lough Furnace. Apart from several fish that did not strongly assigned to any specific cluster (n = 20). I did not find sufficient evidence, either morphologically or genetically, to support the distinction of a partially plated ecomorph among the stickleback sampled in Lough Furnace, as the ecomorph would present a number of lateral plates intermediate to low and completely-plated fish. One explanation for these fish is that they are hybrids, which I discuss in greater detail below.

In Ireland, sticklebacks have been observed in a broad diversity of freshwater habitats and with a high morphological habitat-specific diversity (lake or river, benthic or limnetic) (Ravinet *et al.*, 2013). In that study, strong parallel divergence in key phenotypical traits (body depth, head size, snout size), has been suggested previously to explain a high proportion of morphological variance between marine and freshwater ecomorph pairs. Another study used mitochondrial and microsatellite markers to understand the post-glacial colonisation history of stickleback across the country (Ravinet *et al.*, 2014). That study inferred that the Trans-Atlantic (TA) and the European (EU) lineages have recolonised Ireland after the last maximum glaciation (LGM) in different colonisation events, but it also found evidence for a haplotype unique to Ireland.

This would be the fourth European haplotype identified, in addition to the TA, the EU and the Black Sea (BS) lineages, that have diverged during the late Pleistocene glaciation (Mäkinen & Merilä, 2008). The TA and EU lineages have recolonized freshwater environments in the west of Europe after the LGM. On their global analysis on Ireland, the west coast was mainly composed of the EU lineage, even if there is a strong genetic diversity across Ireland. The results here showed that low-plated freshwater fish from the Burrishoole freshwater habitats (Rough River and Lough Feeagh) were genetically different from low-plated fish found in Lough Furnace. Interestingly, they were the first group to separate from all the others when using only neutral markers in STRUCTURE analysis (Figure 3.4). When analysing the information from the neutral markers separately with a DAPC approach, neutral markers were differentiated mainly individuals based on their environment (freshwater vs brackish-marine), while adaptive markers (more specifically two of them that drive the pattern), differentiated individuals based on their number of lateral plates (Figure 3.5). These results with neutral markers suggest a potential isolation-by-environment as marine samples (from Ireland and the Hebrides) and brackish individuals regardless of their number of lateral plates (low- and completely-plated from Lough Furnace) are more similar than all freshwater samples (Rough River and individuals from Lough Feeagh). Under isolation-by-distance scenario (IBD, when populations are genetically isolated on the basis of geographic distance), the brackish individuals would be expected to be intermediate between purely freshwater and purely marine samples. However, these results suggest an indirect role for adaptation to different salinity in reducing gene flow between population residing in divergent environments. To explain this, it is plausible that after the LGM, marine ancestral sticklebacks likely recolonised Lough Feeagh and surrounding rivers, before being geographically isolated by post-glacial uplift caused by land rebound from the release of

isostatic pressure. There is also a large glacial moraine that also separates the two lakes with the effect of impounding the waters of the freshwater Lough Feeagh. Geographical isolation followed by genetic drift thus seems the more likely process explaining the strong genetic divergence between these Burrishoole sites and the low-plated ecomorph from Lough Furnace, e.g. (DeFaveri *et al.*, 2012).

Elsewhere in its range, stickleback colonisation events have been associated with oscillations in sea level, and Lough Furnace was probably colonised during this period. Results showed that the low-plated ecomorph in Lough Furnace is genetically distinct from Lough Feeagh but also from the completely-plated ecomorph that also breeds in Lough Furnace, indicative of three different populations overlapping in sympatry and parapatry within an area of 1.5 km^2. Lough Furnace is a complex environment with important changes in salinity range, dissolved oxygen, and temperature (Kelly *et al.*, 2018a,b). Therefore, marine ancestral sticklebacks which colonised Lough Furnace probably had to adapt to this new and highly variable environment. Our STRUCTURE results showed that when using only our ten adaptive markers, the first separation for K = 2 occurred between low-plated ecomorphs (Rough River, Lough Feeagh and Lough Furnace) and completely-plated ecomorphs (Lough Furnace and Muingdoran) (Figure 3.6). Moreover, the DAPC results based only on adaptive markers revealed that two loci out of ten were driving the pattern. When removing these two, the eight other loci revealed similar patterns as neutral loci (Figure 3.7). Thus, when considering only the two adaptive loci that were driving the pattern (chrI_21951727, located between two genes associated with salinity tolerance, and chrXIV_11360680, associated with metabolic process), they strongly differentiated low- and completely-plated groups, regardless of their location (Figure 3.8). This suggests that both fish from Lough Feeagh and low-plated from Lough Furnace have been adapting to their newer, freshwater

environments, while completely-plated from Lough Furnace are still genetically closer to the marine populations at specific functional loci. I identified several individuals with a freshwater genotype captured in Lough Furnace (13 out of 1,115 total individuals). These fish may be immigrants from Lough Feeagh, as the samples almost all came from near the two channels that connect the loughs, and which are presumably impassable to upstream migration by stickleback. The potential for such migration is further demonstrated by sticklebacks being observed in salmonid-rearing ponds at the Marine Institute. These ponds source their water directly from Lough Feeagh. I also observed fish with freshwater-type genotypes at other sites in Furnace near other freshwater inputs, such as the Yellow River in the central-eastern part of the lake.

A small number of individuals (n = 26) were identified phenotypically as low-plated during the sampling, whereas their genetic cluster assigned them to completely-plated ecomorph. Most of them (n = 18) were also categorised as juveniles, sampled in June/July and present at the same sampling site as the completely-plated adults. As sticklebacks typically take five months to acquire their full complement of plates, I suspect that these fish are actually completely-plated juveniles yet to grow their lateral plates (Bell, 1981; Banbura, 1989). Of the fish lacking a robust genetic assignment (n = 20), 18 were phenotypically assigned as low-plated and two were assigned as completely-plated. This group was placed between the low-plated and freshwater ecomorphs branch and the completely-plated / marine branch on our dendrogram. These fish appear to be genetically intermediate between low-plated and completely-plated, raising the possibility of them being hybrids. The breeding experiment of Chapter 2 established that hybridisation, at least in one direction, was possible. Chapter 2 also showed reproductive barriers were unlikely to come about via habitat preference or allochrony, suggesting a stronger role for copulatory and post-copulatory barriers to hybrid production. However,

in other systems, studies have shown that hybridisation occurs at very low frequency, probably due to post-zygotic barriers (Jones *et al.*, 2006a), and I suspect the same in our system. An important follow-up to that experiment would be mate-choice trials. Overall, these findings imply that low- and completely-plated ecotypes in the Burrishoole system might be adapted to different ecological niches, even for the low- and completely-pated ecotypes overlapping in the brackish environment (Lough Furnace).

In this study, I used ten SNPs linked to known genetic functions, such as salinity tolerance, digestion, and hormone synthesis, and which had been found previously to significantly differentiate marine and freshwater ecomorphs in other systems (Jones *et al.*, 2012b; Ferchaud *et al.*, 2014a). It is noteworthy that most of these did not differentiate ecomorphs in this system, except two loci associated, respectively, to metabolic process (chrXIV_11360680) and salinity tolerance (chrI_21951727). One other marker (27027), presumed neutral, differentiated ecomorphs based on their location (freshwater versus marine ecomorphs). While this marker did not show evidence for divergence selection in the literature, it is possible that it might be under some divergent selection, or is possibly hitchhiking with other loci that could be under selection. These results add to support generally for the adaptive significance of these two loci in geographically disparate marine versus freshwater pairings. In total, three loci linked to salinity tolerance, showed significant departures from Hardy-Weinberg Equilibrium (HWE) that may be indicative of divergence: for low-plated from Lough Furnace, chrXIV_11360680 linked to salinity tolerance was not at HWE, or for instance in the Muingdoran ecomorph, chrI_21663978 (linked to salinity tolerance) and chrI_21951727 (between two genes linked to salinity tolerance) departed from HWE. These results suggest that even if these adaptive markers are not separating the different ecomorphs to the same degree as in other studies, they still show evidence for some genetic divergence between them. Interestingly,

chrI_21951727 (between two genes linked to salinity tolerance), showed evidence for selection between the low-plated and completely-plated ecomorphs in the BayeScan analysis. These results suggest that after colonising Lough Furnace, stickleback marine ancestors have probably adapted from purely marine to brackish environments. Unfortunately, data for candidate SNPs linked to EDA were not available in this study, as EDA is well known to be highly differentiated between marine and freshwater populations (Colosimo, 2005). However, a previous study Ravinet et al. (2015) which found that EDA-linked microsatellites markers were not segregating the ecomorphs as well as in other systems. Nevertheless, in some systems, EDA has shown unexpected patterns, sometimes the allele giving the completely-plated phenotype is at a very low frequency in the anadromous population occurring in sympatry with a freshwater resident population, suggesting little introgression (Bell et al., 2010). Overall, recent studies have shown that the three-spined stickleback is an excellent model for the study of genomic architecture, especially when marine and freshwater ecomorphs are overlapping and provide a basis for a better understanding of the global pattern of the genomic evolution in ecomorph pairs (Reid *et al.*, 2021; Roberts Kingman *et al.*, 2021).

This study has shown that the different ecomorphs observed in the Burrishoole were genetically distinct from each other and are in substantial agreement with observed morphological differentiation. This study is consistent with previous work suggesting that after the LGM on the west coast of Ireland, sticklebacks have colonised purely freshwater environments first (Rough River and Lough Feeagh), with subsequent colonisation events, linked to the sea level oscillations, accounting for populations such as that of Lough Furnace. These populations, living in allopatry, parapatry or sympatry, have probably diverged genetically by both adaptive and neutral loci under different processes. I did not find strong evidence for frequent hybridisation, but did observe a

small number of instances, suggesting that post-zygotic selection against hybrids may form the main barrier to gene flow between ecomorphs, as only a few individuals (20) out of 1,599 individuals in this study could potentially be identified as hybrids. Some loci were identified as being probably under selection in this system, opening a further avenue to genomic study to identify more markers that separate ecomorphs in this specific system. Our results underscore the value of sticklebacks as a model system in studying the population genetics of adaptive population differentiation, but also show that care is needed when extrapolating from one stickleback ecosystem context to another.

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Supplementary material

Table S3.1. List of the SNPs used in this analysis: Marker_name = name of the marker from the paper, Success = yes is the marker was successfully genotyped, eliminated = one marker was eliminated because of linkage disequilibrium, Adaptive(1) = 1 when the marker was considered adaptive or 0 if considered neutral, Important_gene = marker associated with a gene, Function = the function known of the gene associated, Paper = paper from which the marker is coming from.

Marker_name	Success	Eliminated	Adaptative(1)	Important_Gene	Function	Paper
1139	yes		0		NA	Ferchaud et al. 2014
1800	yes		0		NA	Ferchaud et al. 2014
1955	no		1		elastik fibres and collagen formation	Ferchaud et al. 2014
2113	yes		1		tumor supressor, eye development	Ferchaud et al. 2014
2620	no		0		NA	Ferchaud et al. 2014
4390	yes		0		NA	Ferchaud et al. 2014
5200	yes		1		suppresses cellular proliferation, embryonic development	Ferchaud et al. 2014
6844	yes		1		digestion	Ferchaud et al. 2014
7275	yes		0		NA	Ferchaud et al. 2014
7745	no		1		regulation of epithelial morphogenesis	Ferchaud et al. 2014
9026	no		1		immune responses	Ferchaud et al. 2014
9202	no		1		cell growth and viability	Ferchaud et al. 2014
9202	no		0		NA	Ferchaud et al. 2014
10561	yes		0		NA	Ferchaud et al. 2014

11120	yes	0		NA	Ferchaud et al. 2014
13177	yes	0		NA	Ferchaud et al. 2014
14236	no	0		NA	Ferchaud et al. 2014
15044	no	0		NA	Ferchaud et al. 2014
15728	no	0		NA	Ferchaud et al. 2014
16548	yes	0		NA	Ferchaud et al. 2014
20238	yes	1		hormonal expression	Ferchaud et al. 2014
20574	yes	0		NA	Ferchaud et al. 2014
20825	yes	0		NA	Ferchaud et al. 2014
21643	yes	0		NA	Ferchaud et al. 2014
21858	yes	0		NA	Ferchaud et al. 2014
22319	yes	0		NA	Ferchaud et al. 2014
22693	no	1		transcriptional activators	Ferchaud et al. 2014
26071	no	1		NA	Ferchaud et al. 2014
27027	yes	0		NA	Ferchaud et al. 2014
27608	no	1		crista integrity and mitochondrial function	Ferchaud et al. 2014
28526	no	1		digestion	Ferchaud et al. 2014
28703	yes	0		NA	Ferchaud et al. 2014
31954	yes	0		NA	Ferchaud et al. 2014
32977	yes	0		NA	Ferchaud et al. 2014
33523	yes	0		NA	Ferchaud et al. 2014
35752	yes	0		NA	Ferchaud et al. 2014
5812b	yes	0		NA	Ferchaud et al. 2014
chrI:21487034	no	1	Na/K ATPase9(ATP1a1)	SalinityTolerance	Jones et al. 2012

chrI:21663978	yes		1	Na/K ATPase9(ATP1a1)	SalinityTolerance	Jones et al. 2012
chrI:21689292	yes	yes	1	Na/K ATPase9(ATP1a1)	SalinityTolerance	Jones et al. 2012
chrI:21951727	yes		1	N-acetyltransferase family protein		Jones et al. 2012
chrII:418094	no		1	Mucin_Genes_Family	MucusSecretion	Jones et al. 2012
chrIV:12811933	no		1	EDA	ArmorPlates	Jones et al. 2012
chrIV:12815024	no		1	EDA	ArmorPlates	Jones et al. 2012
chrIV:13931030	no		1			Jones et al. 2012
chrIV:23937349	no		1			Jones et al. 2012
chrXI:5708414	no		1		IonGradient_Resorption_Digestion	Jones et al. 2012
chrXI:5715882	yes		1		IonGradient_Resorption_Digestion	Jones et al. 2012
chrXIV:11360680	yes		1			Jones et al. 2012
chrXXI:5793103	yes		1			Jones et al. 2012

S3.2. Details of the analysis with all markers - STRUCTURE

STRUCTURE analyses carried out on all populations with all markers using the ecomorph prior, showed that the minimum best number of clusters was K = 3 according to the Evanno method. The Log(K) method (Zeisset & Beebee, 2001) suggested a K of 4. For K = 3 groupings determined by STRUCTURE, one group was mainly composed of FEE_LP and RR sampled fish, the second mainly of FUR_LP individuals, and a third comprised FUR_CP, MAR_MU and MAR_HEB (Figure S3.1). These three clusters appear to separate groups based on the number of lateral plates. The K = 4 obtained using the Log(K) method separated further the 'completely-plated' cluster into two groups based on location: one group comprising individuals sampled mainly in Lough Furnace (FUR_CP), the other, the fish sampled at the oceanic and coastal sites, MAR_HEB and MAR_MU respectively. I repeated these analyses excluding MAR_HEB, due to its low sample size (n = 7) and its much greater geographical distance from the other sites. Individuals were assigned to a particular population cluster if they had a STRUCTURE probability for that cluster > 0.8. I identified 13 individuals belonging to the Lough Feeagh genetic cluster that were captured in Lough Furnace, and re-designated these as freshwater type for later analyses. These fish were collected predominantly from the north and east parts of Furnace, near freshwater river inflows (Figure S3.2a). I also identified 26 individuals belonging to the completely-plated Lough Furnace genetic cluster, but which had been designated as FUR_LP based on phenotype. These were small individuals ranging in length from 22 up to 32 mm (with highest of 43.4 mm) and on the basis of their size were categorised as juveniles (Table S3.1), suggesting, based on their genetic

assignment that they were likely to be completely-plated when fully grown. Across all samples, only 20 individuals could not be assigned to a specific cluster based on their low probability scores (probabilities of less than 0.8). Two of these had a maximum assignment probability score > 0.5 to the FUR_CP group, and were treated subsequently as FUR_CP. These unassigned individuals were distributed at different sites across Lough Furnace, from the north of the lake to the estuary (Figure S3.2b). Their second best genetic assignment was to the Lough Furnace low-plated from cluster with probabilities of 0.186 and 0.415 respectively. The 18 remaining unassigned individuals were also assigned genetically to the low-plated Lough Furnace group with an average probability of 0.621 (ranging from 0.210 to 0.798) (Figure S3.2c). Their next highest assignment probability was variously to the low-plated Lough Feeagh or completely-plated Lough Furnace genetic groups. They were distributed broadly across all the sampling sites.

Neighbour-joining analysis, including only individuals with a probability assignment score to cluster of >0.8, indicated a close relationship between individuals collected in the Rough River and the individuals sampled in Lough Feeagh with bootstrap support of 100 (Figure S3.3a). Neighbour-joining analysis also suggested a close genetic relationship between individuals sampled at Muingdoran and the completely-plated fish collected from Lough Furnace with bootstrap support of 92 (Figure S3.3a). The individuals assigned genotypically to the low-plated group in Lough Furnace grouped weakly (bootstrap support) with the completely-plated Lough Furnace and Marine groups, and had an intermediate located on the dendrogram between the completely-plated groups and the freshwater low-plated groups.

When a subsequent neighbour joining analysis (Figure S3.3b) was undertaken which included the 13 Lough Furnace fish that had assigned to Lough Feeagh these putative Feeagh fish based on STRUCTURE also located with strong bootstrap support these fish alongside the Rough River and Lough Feeagh fish. The 20 small FUR_LP fish that assigned with STRUCTURE to the completely-plated genetic group were located on the dendrogram (Figure S3.3b), with fish that are completely-plated from Lough Furnace with a bootstrap of 100, supporting the supposition that these were juvenile completely plated fish. Fish from the Hebrides clustered within the broader completely plated groups. The fish that were unassigned to a group (fish whose maximum STRUCTURE assignment probability was < 0.8) were intermediate in respect of location on the dendrogram found in between the completely-plated and low plated groups, suggesting the possibly of being the hybrid offspring of completely plated and low plated parents.

Table S3.3. Summary of the total length in mm and the number of lateral plates with their standard deviations (SDs) of the different stickleback ecomorphs based on their phenotypic assignment: (Low-Plated: LP, and Completely-Plated: CP), habitat (FEE: Lough Feeagh, FUR: Lough Furnace, MAR_HEB: marine fish from Hebrides, Marine_IRL: marine fish from Muingdoran in Ireland, RR: Rough River).

Ecomorph	Length (mm) ± SD	Plate number ± SD
RR_LP	36.6 ± 7.07	3.3 ± 1
FEE_LP	36.6 ± 8.64	1.8 ± 1.4
FUR_LP	44.4 ± 8.65	5.4 ± 1.7
FUR_CP	65.4 ± 5.77	29.2 ± 1.5
MAR_MU	36.6 ± 16.4	20.8 ± 8.4
MAR_HEB	76.1 ± 10.5	30 ± 1.8

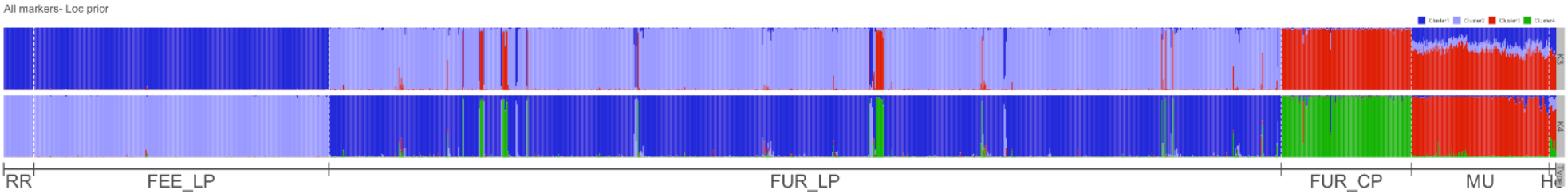

Figure S3.4: All individuals with all markers using the ecomorph as a prior, with their STRUCTURE assignment probability to belong in a cluster, grouped visually by ecomorph: for K = 3 (top) and for K = 4 (bottom).

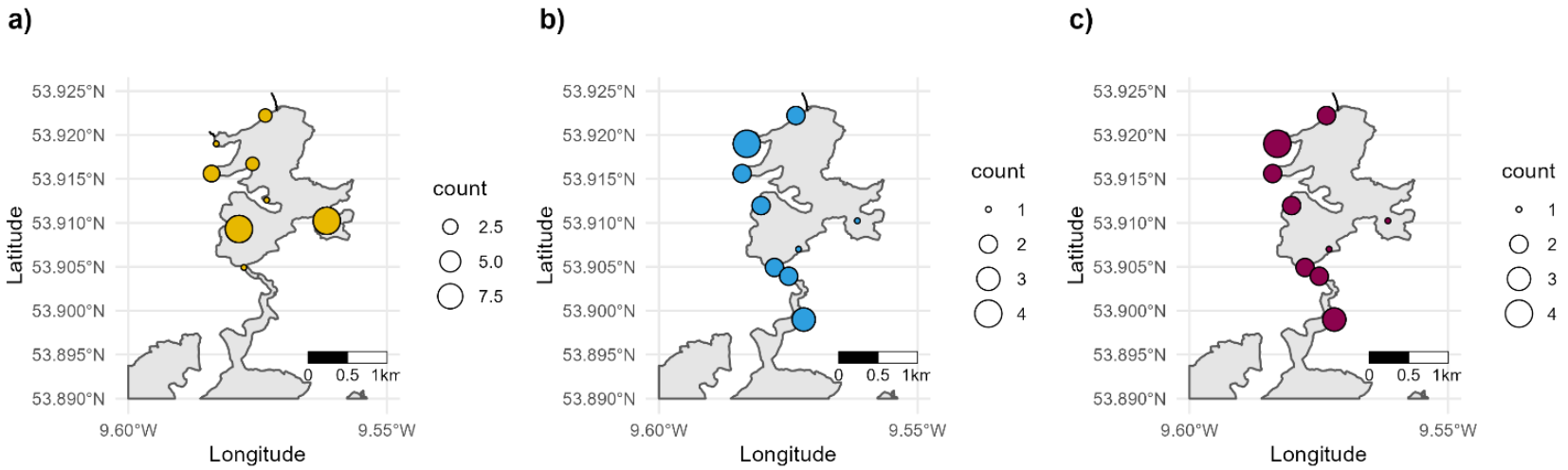

Figure S3.5. Map of Lough Furnace showing the distribution of different groups with unmatched phenotypic and genetic groups: a) Individuals assigned to FUR_LP (based on their sampling location and phenotype) while their genetic cluster assigned them to low-plated Lough Feeagh, b) Individuals assigned to FUR_LP while their genetic cluster assigned them to completely-plated Lough Furnace, c) Individuals that could not be assigned to a specific genetic cluster based on partitioning based on having a maximum STRUCTURE assignment probability > 0.8. Circle size is proportional to the number of fish caught at a given site.

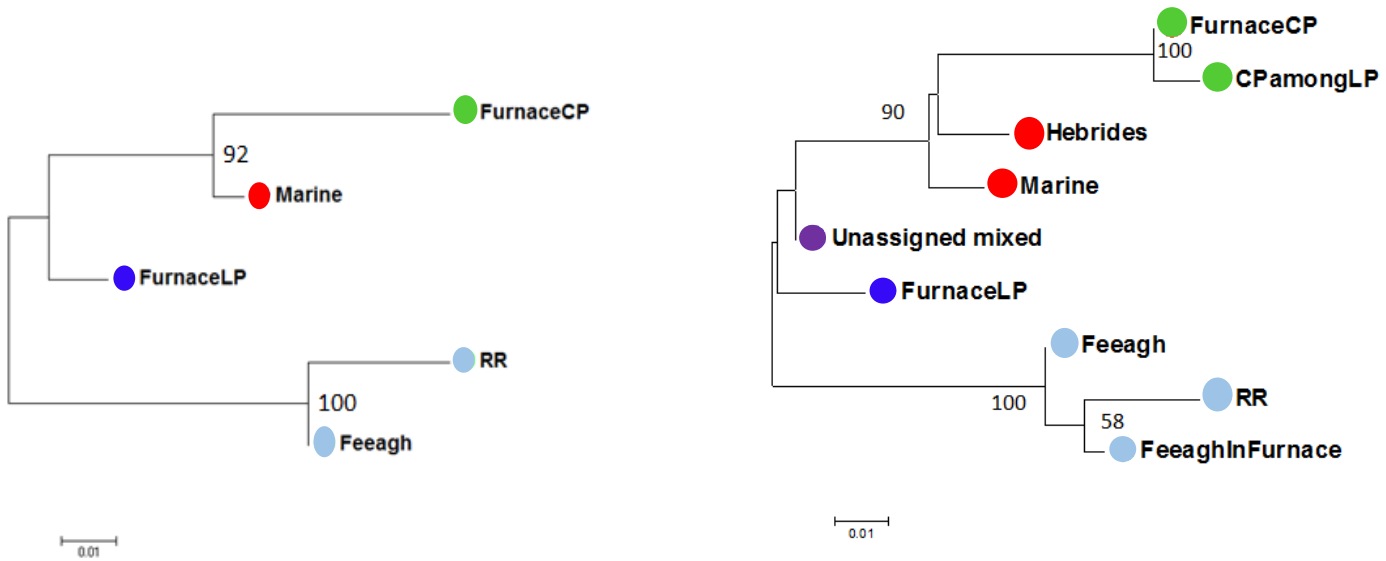

Figure S3.6: Dendrograms representing the distribution of the different groups with their bootstrap support values: a) The five major genetic groups that corresponded with phenotypic groups (Marine Ireland - Muingdoran, completely-plated Lough Furnace, low-plated Lough Furnace, low-plated Lough Feeagh and Rough River); b) As a), but including additional groups for cases where phenotypic and genotypic groups did not match (CPinLP, FeeaghinFurnace, Unassigned mixed).

S3.7. Details of the analysis with all markers - DAPC

All sampling sites

In blind PCA-based K-means clustering, there was no outright best K value. Instead, the analysis suggested a K range of between 1-4. For all samples, as I had four nominal groups based on fish type, the first DAPC axis separated clusters 1 and 4 from 2 and 3, explaining 59.9% of the variance explained while the second axis explains 27.2% of the variance (Figure S4). Assuming a K of 4, testing the degree to which the groups from K = 4 revealed by the DAPC analysis corresponded to the *a priori* groups.

There was very good agreement between the DAPC clusters and the four *a priori* ecomorph groups identified in Chapter 2 (purely marine, completely-plated brackish, low-plated brackish and low plated freshwater) with DAPC cluster 1 (n = 914) predominantly composed of FUR_LP individuals (n = 905; 99.0%), and DAPC cluster 4 (n = 332) mainly composed of FEE_LP individuals (n = 301; 90.7%). DAPC cluster 3 (n = 150) was mainly composed of marine fish (132 MAR_MU and 5 HEB; 91.3%) and DAPC cluster 2 (n = 161) was mainly composed FUR_CP (n = 132, 90%). Axis 1 may thus be considered to separate according to plate type. When DAPC was repeated with ecomorph as a prior, the first discriminant axis explained 46.6% of variance and separated mostly the individuals with a high number of plates (Marine and Furnace completely-plated) from ecomorphs with a low number of plates (Feeagh low-plated and Rough River), with the low-plated from Lough Furnace as intermediate, while the second axis explains 34.6% of variation (Figure 3.2).

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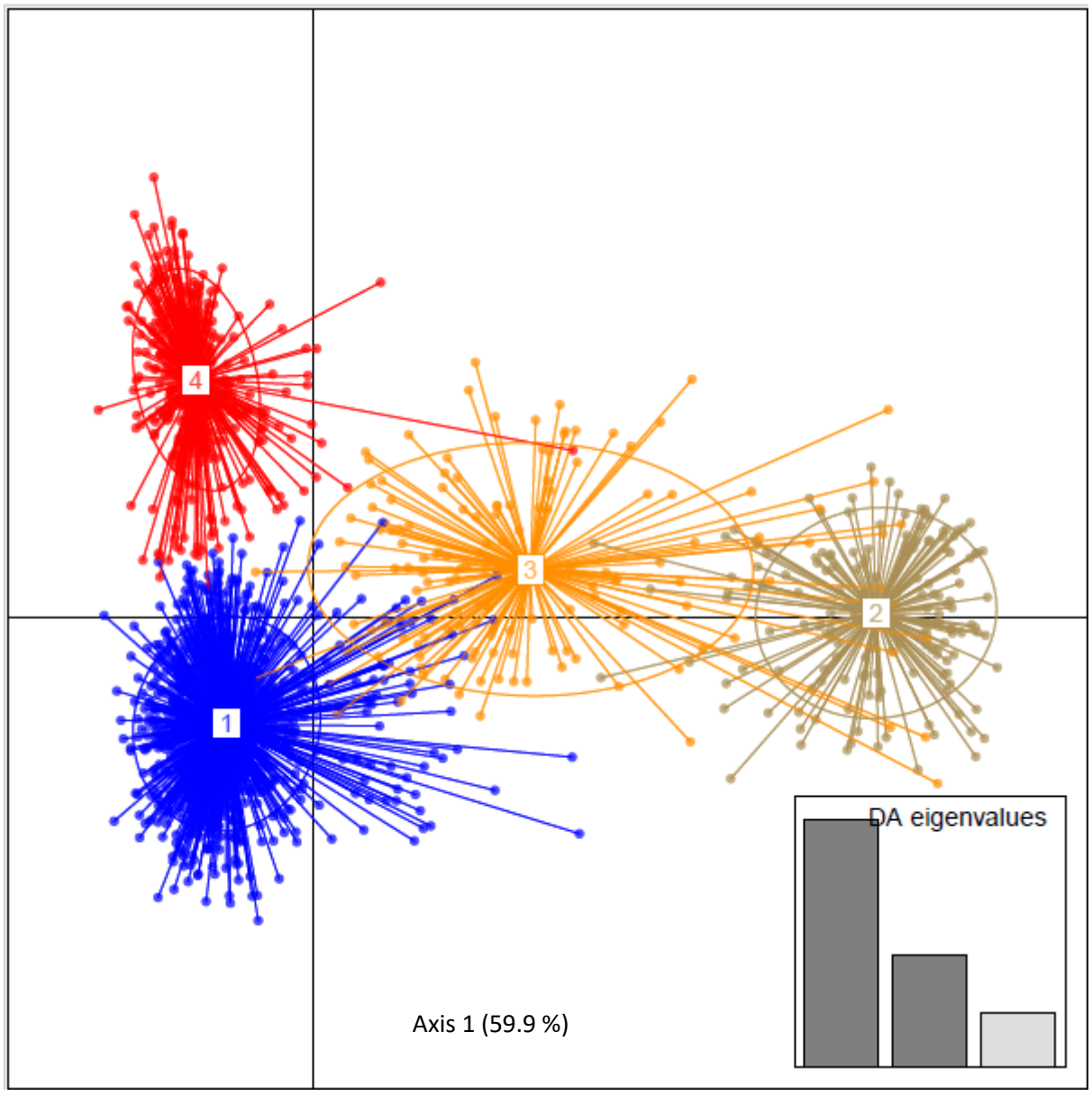

Figure S.3.8: Scatter plots of all samples using the two principal components of DAPC analysis using no prior with K = 4. Individuals are represented as dots and the groups as ellipses. Eigenvalues are also plot in the bottom right box. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

Using K = 3, one group was predominantly composed of FUR_CP, MAR_IRL and HEB, another predominantly of FUR_LP, and the third of RR and FEE_LP (Figure

S5). Again, the first axis (61.5% of variance) can be considered to separate by plate type.

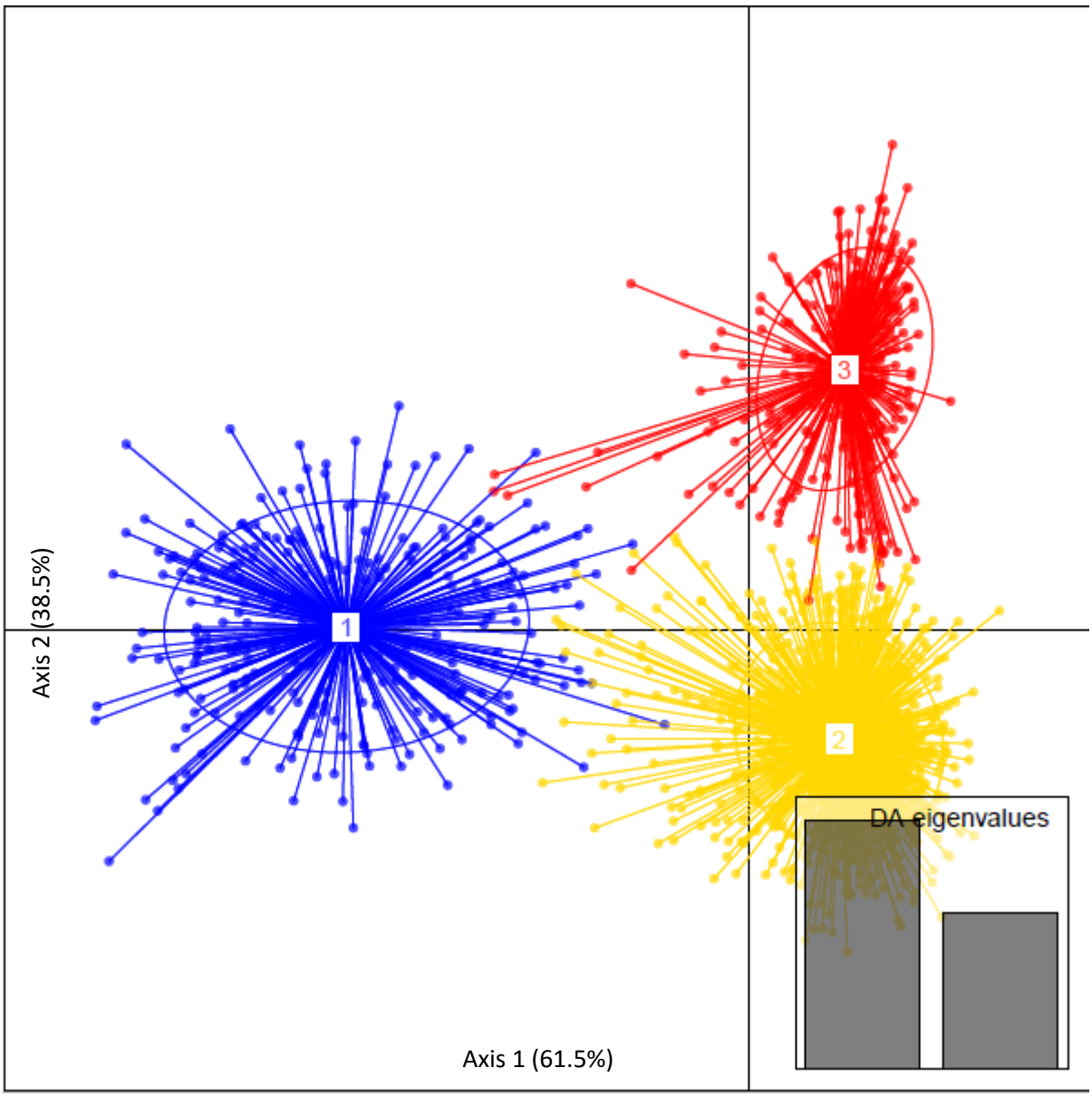

Figure S3.9: Scatter plots of all samples using the two principal components of DAPC analysis using no prior with K = 3. Individuals are represented as dots and the groups as ellipses. Eigen values are also plot in the bottom right box. Numbers in brackets are the proportions of total variance explained by each discriminant function axis.

S3.10. Details of the spatial hierarchical and temporal genetic structure within major location groups analysis with all markers - STRUCTURE

Lough Feeagh

Spatially STRUCTURE analysis of the individuals sampled in Lough Feeagh and the Rough River, the Evanno method suggested a best K of 2 and the log(K) method suggested a best K < 2 (Figure S6). The potential sub-structure in Lough Feeagh seems to be inconsistent across sampling sites.

Among Lough Feeagh individuals, there was low apparent temporal instability as F_{ST} among individuals is low (F_{ST} = 0.010).

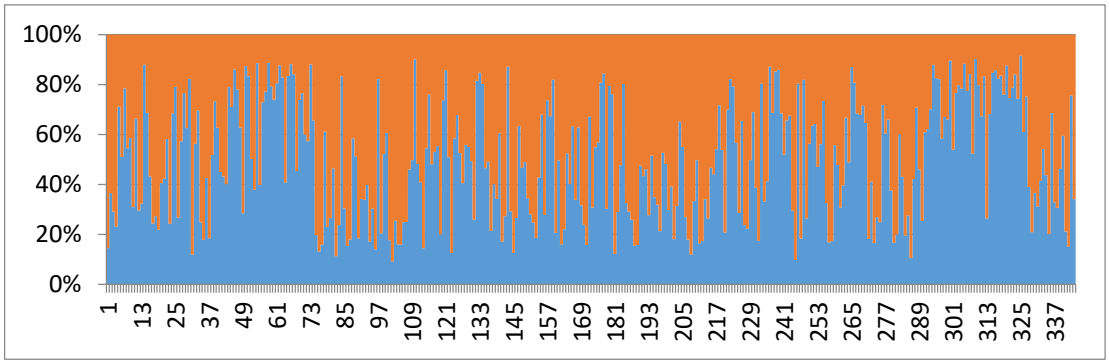

Figure S3.11. Individuals from the Rough River and Lough Feeagh with all markers for K = 2, with their STRUCTURE assignment probability to belong in a cluster. The first 31 individuals are from RR and the other FEE_LP.

Lough Furnace

STRUCTURE analysis on individuals from Lough Furnace suggested that the best number of cluster is K = 2, corresponding to the completely-plated and low-plated ecomorphs. Within each group, STRUCTURE suggested K < 2 for each ecomorph. Among completely-plated and low-plated fish from Lough Furnace, there was low temporal instability in allele frequencies (F_{ST} = 0.016 and F_{ST} = 0.004 respectively).

Marine samples

Among marine samples from Ireland and from the Hebrides, the Hebrides fish appeared to be genetically different from the Irish marine fish sampled at Muingdoran (F_{ST} = 0.051). However, the Hebrides sample size was too low for any strong interpretation to be attached to this (n = 7). For Irish marine fish, the Evanno method suggested that the best number of cluster was K = 2. However, this seemed to be associated with family structuring revealed by COLONY analysis. After, removing the full-siblings from the samples, the best number of cluster was K < 2. Among Irish marine individuals, F_{ST} was relatively high (F_{ST} = 0.044, 95% CI = 0.013-0.082), which might be influenced by the family structuring within the population.

S3.12. Post hoc population genetic summary statistics

Some groups have a low sample size number and results have to be interpreted with caution (7 individuals for the marine fish from the Hebrides and 13 individuals with freshwater (FW) genotype in Lough Furnace (Table 1). All groups presented a mean percentage of total alleles observed per locus superior to 70% ranging to 98% (Table S2). For all groups, the average allele frequency across all locus ranged from 0.54 to 0.6 (Table 2). However, most showed low variability when calculating allele frequency for each locus separately (S6). Some loci were almost monomorphic for the major allele frequency (MAF) in every groups, such as the adaptive 5200 (ranging from 0.89 in one group to completely fixed 1 for six groups) and the neutral 7275 (ranging from 0.82 to completely fixed in four groups) (S7). Some groups show particularly low variability such as: Rough River, Lough Feeagh and FW type in Lough Furnace, which had respectively six, six and seven

almost fixed alleles (observed allele frequency > 0.95), compared to the other groups that had 1-4 fixed loci.

The average observed heterozygotes (H_O) and average expected heterozygotes (H_E), on all markers, were similar or very close for all groups except the phenotypic FUR_LP fish with Lough Feeagh (freshwater) genotypes, which showed a significant deficit in heterozygote ($H_O = 0.09$ and $H_E = 0.12$) (Table S2). Only the Hebrides group (MAR_HEB phenotype and 'marine' genotype) had several loci that showed the highest number with heterozygote deficit or excess (seven with excess and three with deficit). However, this group comprises only seven fish. Other groups with acceptable but relatively low sample size also presented a deficit or excess of heterozygotes. Phenotypic FUR_LP and Lough Feeagh genotype (four loci with a deficit in heterozygotes, n = 13), phenotypic FUR_LP assigned genetically to completely-plated, with two loci having an excess of heterozygotes and five with a deficit (n = 26), and "unassigned individuals" with two loci showing an excess of heterozygotes and five with a deficit (n = 20). No single locus showed consistent patterns of deficit or excess across populations, except for locus chrI_21663978 (linked to salinity tolerance), that presented a deficit in heterozygotes for both Rough River group ($H_O = 0.23$ and $H_E = 0.43$) and the phenotypic FUR_LP fish from the Lough Feeagh (freshwater) genotype ($H_O = 0.38$ and $H_E = 0.49$).

Before correcting for multiple tests (Bonferroni correction), two groups showed significant departure from HWE (fish from Lough Feeagh and Irish marine fish collected at Muingdoran) (Table S7). After Bonferroni correction, only the Irish marine group showed significant departure from HWE ($P < 0.001$). When considering each group and each locus separately, the Irish marine fish had six loci

that departed significantly from HWE before correcting for multiple tests. However, after correction, only two were still significant: chrI_21663978 (linked to salinity tolerance) and chrI_21951727 (physically linked to two genes linked to salinity tolerance). In the Lough Feeagh genetic group, two loci departed significantly from HWE even after correction (27027 and 20825, both neutral), and two others departed significantly before correction (6844 and chrXIV_11360680). No other within-group instances of significant departure from HWE were detected.

Table S3.13. Summary of different statistic index per group: n is the number of sample per group, Al_pr is the percentage of total alleles observed per locus per population, Al_fre is average of the major allele frequency per group, H_O is the average proportion of observed heterozygotes, H_E is the average proportion of expected heterozygotes and HWE is the uncorrected p-value for deviation from HWE. : The ecomorph group are the following: RR = Rough River; FEE_LP = fish from Lough Feeagh; FUR_LP = low-plated fish from Lough Furnace; FUR_CP = completely-plated from Lough Furnace; MAR_MU = Irish marine fish; MAR_HEB = marine fish from the Hebrides; FWinFUR = fish with freshwater (FW) genotype in Lough Furnace; CPinLP = completely-plated genotype group assigned as low-plated fish from Lough Furnace by phenotype; and UNK = unassigned fish.

	RR	FEE_LP	FUR_LP	FUR_CP	MAR_MU	MAR_HEB	FWinFUR	CPinLP	UNK
n	31	304	924	132	142	7	13	26	20
Al_pr	73.3	93.33	98.33	98.33	98.33	86.67	68.33	83.33	95.0
Al_fre	0.60	0.60	0.58	0.55	0.60	0.59	0.59	0.54	0.58
H_O	0.14	0.17	0.27	0.21	0.26	0.29	0.09	0.19	0.22
H_E	0.15	0.19	0.27	0.21	0.27	0.27	0.12	0.19	0.25
HWE (P)	1	0.0005	0.117	0.599	0	1	1	1	0.93

When comparing F_{ST} between pairs of groups, the highest were between CPinLP group with Rough River and Lough Feeagh (0.51 and 0.44 respectively), followed by the comparison between completely-plated from Lough Furnace and Rough River and Lough Feeagh (0.47 and 0.41 respectively) (Table S3). Then, Muingdoran and Hebrides marine groups showed the highest value when compared to Rough River and Lough Feeagh (range from 0.28 to 0.39). The lowest F_{ST} value was between completely-plated Lough Furnace and CPinLP with 0.0008, followed by FWinFUR and Lough Feeagh (0.025). Also, Rough River and Lough Feeagh had a low F_{ST} (0.062); similarly, the Irish marine group and Hebrides group had a F_{ST} of 0.054.

Table S3.14. Summary of F_{ST} value: in the lower diagonal, actual overall F_{ST} values and in the upper diagonal, 95% confidence interval for each F_{ST} (lower-highest). The ecomorph group are the following: RR = Rough River, FEE = fish from Lough Feeagh, FUR_LP = low-plated fish from Lough Furnace, FUR_CP = completely-plated from Lough Furnace, MAR_IRL = Irish marine fish, MAR_HEB = marine fish from the Hebrides, FWinFUR = fish with freshwater (FW) genotype in Lough Furnace, CPinLP = completely-plated genotyped fish categorised as low-plated fish from Lough Furnace and UNK = unassigned fish.

	RR	FEE	FUR_LP	FUR_CP	MAR_MU	MAR_HEB	FWinFUR	CPinLP	UNK
RR	#	0.043-0.097	0.235-0.276	0.456-0.490	0.283-0.333	0.366-0.492	0.101-0.298	0.487-0.563	0.207-0.317
FEE	0.062	#	0.175-0.187	0.401-0.424	0.260-0.300	0.251-0.363	0.013-0.087	0.411-0.465	0.094-0.205
FUR_LP	0.251	0.179	#	0.262-0.280	0.167-0.193	0.139-0.240	0.155-0.194	0.272-0.303	0.043-0.777
FUR_CP	0.465	0.409	0.269	#	0.193-0.230	0.130-0.308	0.353-0.401	0.0002-0.036	0.1756-0.278
MAR_MU	0.306	0.278	0.178	0.208	#	0.051-0.122	0.210-0.264	0.204-0.251	0.079-0.154
MAR_HEB	0.389	0.279	0.161	0.175	0.054	#	0.262-0.428	0.170-0.333	0.061-0.2
FWinFUR	0.158	0.025	0.168	0.373	0.235	0.284	#	0.393-0.471	0.077-0.190
CPinLP	0.510	0.434	0.286	0.001	0.217	0.194	0.416	#	0.185-0.304
UNK	0.241	0.134	0.045	0.217	0.102	0.079	0.094	0.228	#

Chapter 4 : Ecological and life-history predictors of metabolic trait variation in wild-caught three-spined stickleback (*Gasterosteus aculeatus*)

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Amendments post-via

Additional analyses were carried out on this chapter post-viva, and provide a stronger statistical basis for my already-cautious interpretation of the confounding of the marine ecomorph with high-salinity treatments, they do not produce interpretations that diverge from the original submission.

Abstract

Metabolic rates are fundamental components of life-history strategies. In understanding the processes that they shape and are shaped by, species with strong intraspecific variation in traits such as migratory tendencies, dietary preferences, and reproductive behaviour, are invaluable assets. In the present study, I measured metabolic traits in wild-caught three-spined stickleback (*Gasterosteus aculeatus*), a model species in evolutionary biology, known for its multiple, independent colonisation events of freshwater from marine sources and for its high intraspecific variation. I tested a set of candidate biotic and abiotic predictors of four metabolic phenotypic traits in fish of three different ecomorphs out of four identified in this system: purely marine completely-plated, brackish completely-plated, and brackish low-plated. Marine, completely-plated, fish had significantly higher standard metabolic rate (SMR) than brackish-sourced fish of either plating type, while the two brackish-sourced groups did not differ significantly. Sex was not a significant predictor of SMR, but juvenile marine individuals had significantly higher SMR than any other group, and males expressing nuptial coloration showed significantly higher SMR than males without coloration, suggesting breeding as indicated by coloration 'costs' a portion of male aerobic scope. Warmer experimental temperatures within the study populations' naturally experienced ranges were associated with higher SMR but not maximum metabolic rate (MMR). Experimental manipulation of salinity showed significant, positive relationships with both SMR and MMR. When set against previous respirometry work on three-spined stickleback, our results underscore the difficulty in drawing general conclusions in this species. Disagreements and agreements between studies may come from often strongly-contrasting experimental designs, but may also reflect the complex, often location-specific, evolutionary history

of this species. Future use of sticklebacks for investigating the evolution of fish metabolic traits may require the development of experimental designs that target points of divergence between existing studies

Keywords: Ecomorph, metabolic rate, respirometry, salinity, stickleback

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Introduction

Metabolic rates of organisms are critical to a broad suite of life-history and ecological traits (Pettersen *et al.*, 2016), such as growth rate (Zeng *et al.*, 2017), survival (Bochdansky *et al.*, 2005) and reproduction. Measuring metabolic rates is thus key to understanding the energetic costs of an organism in its natural environment.

A common measure for metabolic trait is standard metabolic rate (SMR), the minimum rate of energy consumption when the organism is unstressed, non-reproductive, non-digestive and inactive (Chabot *et al.*, 2016). Numerous species display significant inter-individual variation of SMR known to be associated with potential behavioural or physiological trade-offs (McCarthy, 2000; Nespolo & Franco, 2007; Broggi *et al.*, 2009; Burton *et al.*, 2011). For example, individuals with a higher metabolic rate may grow faster or be more physically active (Priede, 1985; Álvarez & Nicieza, 2005). Environmental factors can affect these trade-offs, such as via resource limitation or stress exposure (Archer *et al.*, 2021). For instance, European sea bass juveniles (*Dicentrarchus labrax*) show a stronger relationship between SMR and risk-taking while foraging when food-deprived (Killen *et al.*, 2011), and increase their activity and metabolic rate when exposed to mild and severe hypoxia (Killen *et al.*, 2012a). In deer mice (*Peromyscus maniculatus*), individuals with higher metabolic rate, or with the capacity to increase it, were better able to support activity (foraging and exercising in a wheel) under cold temperatures (Sears *et al.*, 2009).

Once corrected for allometric relationships with body mass (Steyermark *et al.*, 2005), SMR shows substantial variation among individuals within the same species and population (McCarthy, 2000; Burton *et al.*, 2011). In fish, this variation can be up to a

factor of three for individuals of the same sex and age (Norin & Malte, 2011; Killen *et al.*, 2012b), making it a good trait for studying within-species and within-population life history variation (Metcalfe *et al.*, 2016). Fish metabolic rate is also strongly influenced by environmental conditions such as temperature (Brown *et al.*, 2004), and varies among different fish ecologies and life-history strategies (Killen *et al.*, 2010). For example, in Atlantic salmon (*Salmo salar*), fish that migrate to sea at a younger age tend to have a higher SMR than those migrating at an older age (McCarthy, 2000). Similar patterns have been observed on other species: in rainbow trout (*Oncorhychus mykiss*), females with a higher SMR have a higher rate of facultative anadromy than females with a lower SMR (Sloat & Reeves, 2014); similarly for brook trout (*Salvelinus fontinalis*), anadromous individuals showed higher metabolic rates than resident individuals (Morinville & Rasmussen, 2003). In capelin (*Mallotus villosus*) in the Barents Sea, metabolic cost was related to migratory behaviour (Behrens *et al.*, 2006) and reproductive status, with SMR being ~30% higher for pre-spawning females compared to post-spawning females (Karamushko & Christiansen, 2002). Such relationships between reproductive status and SMR are not unique to fish: for example singing male Carolina wrens (*Thryothorus ludovicianus*) can demosntrate 2.7-8.7 times their basal metabolic rate (Eberhardt, 1994), and male wax moths (*Achroia grisella*) that produce ultrasonic signals can have up to four times the SMR of non-reproductive males (Reinhold *et al.*, 1998).

SMR is only one component of an individual's metabolic phenotype. Another component is maximum metabolic rate (MMR), when the organism is at its maximum of energy consumption under stress (Norin & Clark, 2016). A routine example of such a stress is acute, intense physical activity. The difference between SMR and MMR describes the aerobic scope (AS), a proxy for the capacity to use energy for functions such as locomotion, growth, digestion and reproduction, once the baseline energy demand has

been satisfied (Guderley & Pörtner, 2010). MMR and AS show similar levels of intraspecific variation to those seen in SMR (Metcalfe *et al.*, 2016), and are themselves important for life history. For example, variation in MMR and AS have both been linked to swimming performance in the three-spined sticklebacks (*Gasterosteus aculeatus*) (Tudorache *et al.*, 2007), migration distance capacities in the sockeye salmon (*Oncorhynchus nerka*) (Eliason *et al.*, 2011), and aggressive and dominance behaviours in tropical damselfish (*Pomacentrus amboinensis*) (Killen *et al.*, 2014). MMR and physical performance can predict a male's courtship quality (Clark, 2012), with examples including quality of defence of territory in the spider *Agelenopsis aperta* (Riechert, 1981), or the quality of nest-building three-spined stickleback (Ward *et al.*, 2004).

Migrating between environments requires coping with different environmental condition. Euryhaline fish (fish that are able to thrive in a broad range of salinity) must adjust their osmoregulation when salinity changes (Moyle & Cech, 1996), but adjusting osmoregulation has a cost. In Chinook salmon (*Oncorhynchus tshawytscha*), and the resident and anadromous forms of *O. mykiss* ('rainbow' and 'steelhead' trout respectively), salinity change experiments on fish previously acclimated to a range of different salinities found that all showed an increase in metabolic rate when exposed to higher salinities (Morgan & Iwama, 1991). Measuring the variation in different aspects of metabolic phenotypes, like SMR, MMR and AS, at different salinities in fish that routinely experience salinity changes may thus be a good proxy for estimating the cost of osmoregulation (Fry, 1971).

Another major pressure affecting metabolic phenotype is predation risk, and especially the ability to move sufficiently fast and far to escape a predator's attack. Strategies for escaping predator attacks are trade-offs between escaping too early and missing foraging opportunities, and delaying escape but increasing the risk of being captured (Ydenberg

& Dill, 1986; Krause & Godin, 1996; Bohórquez-Herrera *et al.*, 2013). In such scenarios, a key component of metabolism is recovery time, the speed with which high-O_2 consumption can be reduced back to a more routine level. In fast-start exercise, recovery time too, shows strong intraspecific variation (Marras *et al*., 2010; Killen *et al*., 2014). Slower recoveries may thus be costlier in terms of net energy expenditure. A study showed that AS is related to the ability to recover from burst-type anaerobic activity (Killen *et al.*, 2015), and that individuals that are slower to recover may be less reactive to stimuli that would elicit an anaerobic burst response than individuals which recover faster (Marras *et al*., 2010; Killen *et al*., 2014). Measuring the AS and the recovery time may thus provide insights on anti-predator behaviours. As with other metabolic traits, the trade-off between energy expenditure and readiness for further action may be further complicated by environmental variables (Suski *et al*., 2006).

The three-spined stickleback (*Gasterosteus aculaetus, L. 1758*) is a small teleost fish distributed across the temperate and subarctic zones of the Northern Hemisphere (Wootton, 1984), and has become a model species to study evolutionary biology and behavioural ecology (Ostlund-Nilsson *et al.*, 2006; Huntingford & Ruiz-Gomez, 2009; Barber & Nettleship, 2010). Ancestral marine populations have repeatedly colonized freshwater lakes and rivers after the retreat of glaciers during the Pleistocene, leading to the rapid, parallel divergence of many stickleback ecomorphs from their ancestral marine ancestors (Bell & Foster, 1994). One trait that has gathered particular interest is the loss, completely or partially, of lateral plate armour in freshwater populations (Bell *et al.*, 2004; Barrett *et al.*, 2008). These plates are thought to play an important role as a defence against predators (Reimchen, 2000). Three different ecomorphs are commonly recognized based on their plate phenotype (Münzing, 1963; Hagen & Gilbertson, 1973): completely-plated, observed in marine and brackish environment; partially-plated, found

in brackish environments; and low-plated, found in freshwater and brackish environments. Stickleback can thrive in a broad range of salinity (Wootton, 2009), from the purely freshwater resident to the anadromous ecomorph that migrate in brackish environment to breed and move back to the ocean afterwards, with a complex and broad behavioural range (Kitano *et al.*, 2012). These intraspecific phenotypic contrasts make sticklebacks an excellent model species for studying the relationships between metabolic traits and life-history trade-offs. Previous work on stickleback metabolism in relation to migratory phenotype have reported higher metabolic rate for anadromous compared to the freshwater resident ecomorphs: stream-resident individuals have shown a reduced capacity for prolonged swimming compared to marine individuals (Taylor & McPhail, 1986; Tudorache *et al.*, 2007), and that MMR is a good predictor of between-individual variation in prolonged swimming capacity (Dalziel *et al.*, 2012b). Other stickleback metabolic research has found that SMR and AS are significantly lower at cooler temperatures (Ressel *et al*., 2021) but that salinity does not seem to have an effect on SMR when fish are acclimated to different treatments (Grøtan *et al.*, 2012).

The aim of this study is to characterise variation, and test for predictors of, a set of metabolic traits for three different three-spined stickleback ecomorphs: marine-resident completely-plated (from Muingdoran), brackish-anadromous completely-plated, and brackish-resident low-plated (both from Lough Furnace). I measured SMR for all fish and, when possible, MMR, AS and recovery time (T_R). I also performed an experiment with simulated tide conditions for low-plated Furnace ecomorphs by increasing or decreasing salinity within a range they can experience in the wild. I hypothesized that: (1) sticklebacks from different locations and different phenotypes have different metabolic rate under similar environmental conditions (SMR, MMR and AS); (2) SMR increases with abrupt salinity change for low-plated brackish resident fish; (3) recovery

time is higher for the low-plated resident ecomorph than for the completely-plated anadromous ecomorph.

Methods

1. Study area

Sticklebacks were sampled from Lough Furnace (N53°55′22″, W9°34′20″) and from Muingdoran (N54°10′30″, W9°99′04″), both in the west of Ireland. These locations are 50 km apart, and the probability of gene flow between them, in the absence of evidence to the contrary, is low. Lough Furnace is a brackish, tidal lagoon in the intensively-studied Burrishoole catchment (Whelan *et al.*, 1998; Cassina *et al.*, 2013; de Eyto *et al.*, 2020). The lagoon is deep and permanently stratified, with a strong, shallow pycnocline (~2.4 m), high dissolved oxygen concentrations at the surface, and anoxia below ~6-7 m. During spring and summer, the temperature also changes dramatically, with warmer water above the pycnocline (15-22°C) and a strong decrease below the pycnocline (11.6°C). Conversely, in winter, temperature is stable under the pycnocline (~11.6°C, similar to summer), whereas the temperature surface decreases (3-8°C). The lake is known to experience occasional, near-total mixing events that can drastically change conditions in a very short time: temperature and oxygen can change in a single day, and strong winds can cause important upwelling events in the lake (Kelly *et al.*, 2018a,b). In terms of salinity, Lough Furnace is essentially marine below the pycnocline (~22 PSU). At the surface, salinity is low to absent (0-4 PSU) in the north part of the lake where major freshwater inflows occur, moving to brackish concentrations (10 to 15 PSU) in the channel that connects to the estuary in Clew Bay, which connects directly to the Atlantic Ocean. At Muingdoran, salinity fluctuates around marine (22-36 PSU). Muingdoran is

composed of many purely marine rock pools along the shoreline. These rock pools are directly connected to the Atlantic Ocean at high tide, but are cut off at low tide.

2. Sampling from wild populations

From January to October 2019, unbaited minnow traps (silver galvanized Gee-type model #1279 Frabill, Jackson, Wisconsin, USA) were set at one or two of five sampling sites in Lough Furnace (with alternative sites sampled on each sampling occasion), and in different nearby rock pools (considered as one sampling site) at Muingdoran. Traps were set from 1-2 m of the shoreline at 0.5-2.0 m depth in Lough Furnace, and in the middle of the rock pools of depth < 1 m in Muingdoran. Traps were set in the early morning and collected the next day, subject to tides. Salinity and temperature were measured at each site upon collection of traps (WTW Multi 340i Handheld Multimeters, Germany). At each sampling, only six fish were collected. As temperature and salinity conditions can vary strongly from one rock pool to another, fish were collected from one pool per visit, or from two pools in close proximity verified by instruments as having the same conditions at that time. From Lough Furnace, I sampled only adults, as juveniles were too small to assign to a plate phenotype category and difficult to assay with our respirometry equipment. At Muingdoran, I sampled a mixture of adults and juveniles, owing to the relatively low abundance of adults, coupled with juveniles being bigger than Lough Furnace juveniles and reliably assignable as “marine completely-plated”. In August at Muingdoran, I only sampled juveniles, as adults had left the rock pools. Sexual maturity was estimated by eye in the field based on fish size, and confirmed during laboratory dissection by observation of gonads. Individuals captured outside the breeding period were categorised after gonad inspection and phenotypic measurements. After capture, fish were fasted for 24 hrs in a dark and quiet room, in a 50:50 mixture of water

from the capture site and water from the respirometer system (see below). Salinity of the water from the respirometry system was adjusted to match the capture site to the nearest 1 PSU.

3. Experimental design

SMR was measured using an automated intermittent flow respirometry system. The experimental setup comprised of eight glass cylinder chambers (Inner Diameter (ID) 84 mm, 49.35 mL and ID 104 mm, 65.89 mL) housed in two separate tanks (40 L) of dechlorinated, UV-filtered tap water. For individuals coming from Muingdoran, freshwater was mixed with sea salt free from added iodide or anticaking agent (BuyWholefoodsOnline, UK) to reach the same salinity as in rock pools. Each cycle of intermittent flow respirometry consisted of an 'open' phase, in which air-saturated water from the water bath was flushed through the chambers, evacuated back into the bath via outflows opening above the water surface, and a 'closed' phase in which the chambers were sealed and each chamber's water was moved over an oxygen sensor (see below) in a recirculation loop. Temperature was controlled by a thermostatic relay linked to external heating and cooling sumps. The setup simultaneously recorded the temperature of the water baths and the oxygen consumption in the eight chambers using oxygen probes (Ocean Optics, Dunedin) connected via a fibre optic cable to a Witrox 4 device (Loligo Systems, Viborg, Denmark). Fish were randomly allocated to chambers, with one randomly allocated empty chamber per bath for measuring background bacterial respiration. Fish were left overnight in the chambers, in a dark, quiet, isolated room, and percentage oxygen saturation recorded every second until the next morning with periods of 720-900 secs of chamber closure, alternated with flushing periods of 90 secs. I used Loligo Systems AutoResp software (v2.2.0) to collect and save the data from the Witrox

devices, and to control the pumps for the intermittent-flow cycles. In all analysis, the first 2-3 hrs were systematically removed to ensure that fish were settle down after handling.

MMR measurements were taken in the late morning or early afternoon on the day following SMR measurement. Each fish was manually chased in a 4 L bucket for 2 mins to achieve a standardised, high, non-injurious exhaustion (no response to tactile stimulus and no burst swimming behaviour, but no loss of swimming equilibrium) following best practices (Norin & Clark, 2016). This method produces MMR results comparable to those of swimming chambers (Killen *et al*., 2007). After this time, each fish was immediately placed into its respirometer chamber and the oxygen consumption was measured for 5 mins. The average time to transfer the fish was 10 secs and never exceeded 25 secs. After MMR, automated intermittent-flow measurements were resumed for 1-5 hrs to assess the recovery time (T_R), defined as the time required for the oxygen consumption to return from the maximum after MMR to 50% of the total AS (Marras *et al.*, 2010; Killen *et al.*, 2014). Due to variation in T_R and constraints on personnel and shared equipment, not all fish achieved recovery in the available time. When each respirometry run was over, fish were removed from the chamber and euthanized with an overdose of MS-222 (Tricaine methanesulfonate, FVG Ireland), and all equipment was bleached and rinsed.

4. Experimental protocol for salinity response

For 61 fish, a salinity change experiment was performed to measure the metabolic response under simulated tidal conditions. After measuring SMR and MMR, the salinity of one tank containing four chambers was changed in one of two scenarios, while the other was held constant. The first scenario was an increase of the salinity from a purely freshwater or very low salinity (0-5 PSU) to brackish salinity (15 PSU), and the second

was a decrease of the salinity from a brackish salinity (15 PSU) to low salinity (4 PSU). These salinity ranges are based on the minimal and maximal salinity measured at the respective sampling sites during trap placement and collection (Table S4.1). These ranges likely reflect the real-world experience of adult sticklebacks in Lough Furnace during the breeding season, when both sexes stay within the shallow water in which males build and defend nests. For both the experimental and control tanks, 8 L of water was removed, and then replaced with water of appropriate salinity to achieve the desired final salinity. Probes in salinity-change experiments were calibrated for freshwater (0 PSU), with oxygen saturation later adjusted for salinity using the *convert_rate* function of the RespR package (Harianto *et al.*, 2019). Fish were kept for an extra 24 hrs in the chamber to measure the potential shift of oxygen consumption under new salinity conditions. This research was approved by the animal ethics and welfare bodies of University College Cork and the Marine Institute, and conducted under Health Products Regulatory Authority (HPRA) license number AE19130-P096.

5. Phenotypic measures

In total, 144 three-spined sticklebacks were sampled and used for respirometry experiments (see 2 c.). Lateral plates were counted under a binocular magnifier (Stereo Binocular, Euromex, magnification 0.7-4.5×). Fork length and total length were measured to the nearest mm using callipers after the experiment. Wet mass was measured to the nearest 0.1 g. A small fin clip (ca. 2 mm^2) was taken and preserved in absolute ethanol. Sex was determined during dissection by inspecting gonads. Individuals that could not be assigned to female or male (that were not juveniles anymore but not sexually mature yet) were removed for downstream analysis. Male coloration status (red-orange

throat and/or blue eyes) was recorded, and for females, the presence or absence of eggs was recorded.

6. Metabolic trait estimation

Raw data from AutoResp were processed for analyses in the statistical software R v4.1.0 (R Core Team, 2018), using the package RespR package v1.1 (Harianto *et al.*, 2019). Per-cycle oxygen consumption rates were estimated using the *auto_rate* function in RespR. This function uses a customisable rolling regression method to identify the longest, 'most linear' section of an oxygen consumption slope. Oxygen saturation slopes were inspected, and 14.1% of cycles were removed for downstream analysis for having $R^2 < 0.9$. Oxygen saturation slopes were then converted to mass-specific oxygen consumption (MO_2) in mg/kg/hrs, all corrected for background respiration based on the decline in saturation recorded in the empty chambers. SMR was then calculated as the mean of the lowest 10^{th} percentile of MO_2 values. MMR was estimated following the same method as SMR: use of the *auto_rate* function over the 5 mins of measurement after chasing, and adjustment for background respiration per run. Absolute AS was calculated as the difference between MMR and SMR.

7. Statistical analysis

All statistical analyses were performed in R, and all plots were produced with R ggplot2 package v 3.3.5 (Wickham, 2016). All analyses conformed to their respective assumptions unless otherwise stated.

For SMR, three individuals were removed from downstream analyses for showing atypical 'settling' signatures (metabolic rate did not decline from the high values at time of loading to chambers), along with three individuals with unrealistically and

discontinuously low estimated SMR values (all < 10 mg/hr/g; next value > 14 mg/hr/g). Marine fish were excluded from MMR analyses, as the sample size was too low after excluding fish showing adverse reactions during chasing or MMR values suggestive of adverse reactions (n=5). In fish caught in Lough Furnace, one with an extremely and unrealistically high MMR value was also removed (>1000 mg/h/gr). A subset of 58 individuals all from the Furnace low-plated group (used in SMR) were kept for the salinity change experiment models: 17 individuals with an increase of salinity, 12 individuals with a decrease of salinity, and 29 no-change 'control' individuals. AS was calculated for all individuals with a retained MMR value. Recovery time (T_R) was analysed as a survival-type variable. Only experiments with at least two fish that recovered by the end of the experiment were kept for the analysis, resulting in a total of 44 individuals (13 completely-plated and 31 low-plated from Furnace). Fish used for the salinity change experiment were not analysed for T_R.

With the exception of T_R (see below), all statistical models used were linear mixed model performed with the *lmer* function from the lme4 package (Bates *et al.*, 2015). Mass was Z-transformed in all models. A variable combining water bath number (one or two) and the date of the experiment was used as a random effect in all models. Sensor probe number was tested as an additional candidate control predictor, but was not supported by AIC_C. For fish with two SMR measurements as part of the salinity change experiment, only day 1 data were used. Salinity and temperature were tested with and without the marine completely-plated group for SMR. To test for a potential causal relationship between salinity and SMR, the salinity analysis was further repeated on just fish that were measured on two separate days as part of the salinity change experiment, using both days' measurements and with the addition if fish ID as a random effect, and controlling for the direction of salinity change applied. This was followed up by testing for the effect

of significant between-individual variation in response to salinity by the addition of a random-slopes term. T_R was analysed using survival analysis with the *survreg* function from the R package 'survival' (Therneau & Grambsch, 2000), using right-hand censoring to accommodate individuals that did not reach their recovery threshold in the time allowed. I tested ecomorph and sex variables separately, and compared models with exponential and Weibull distributions and compared them with AIC_C. In a distinct model with males only, I also tested among the males if the presence of nuptial coloration had an effect on recovery time. Female egg status was not tested as a predictor of T_R because only two females without eggs were assayed.

Results

1. Metric and metabolic traits diversity in the sticklebacks

Mass, length and plate number are summarised by ecomorph, capture location and sex in Table 4.1. In general terms, these describe patterns reflecting previous stickleback work: females tended to be longer and heavier than males, but to have similar numbers of plates; and marine completely-plated fish tended to be longer and heavier than brackish completely-plated fish, which were, in turn, longer and heavier than brackish low-plated fish. Marine juveniles (individuals that were not sexually mature) were smaller and lighter than the marine adults.

Table 4.1. Summary of phenotypic traits mean values associated with their standard deviations (SDs) of the different stickleback ecomorphs (FUR_CP: Furnace completely plated, FUR_LP: Furnace low-plated, Marine_CP: Marine completely-plated), sex (female, juvenile, male), the number of individuals per group for SMR and for MMR in brackets, the body mass mean per group (gr), the mean length per group (mm) and the mean plate number per group.

Ecomorph	Sex	N for SMR (MMR)	Body mass (gr) ± SD	Length (mm) ± SD	Number of plate ± SD
FUR_CP	female	8 (8)	2.62 ± 0.89	67.2 ± 2.96	30.4 ±1.27
FUR_CP	male	12 (12)	1.96 ± 0.45	59.2 ± 5.31	29.5 ±1.45
FUR_LP	female	40 (37)	1.26 ± 0.48	50.5 ± 5.67	6.06 ±0.83
FUR_LP	male	42 (42)	0.88 ± 0.44	45.4 ± 4.6	5.99 ±0.89
Marine_CP	female	5 (0)	3.24 ± 0.46	68.4 ± 3.57	30.0 ±0.79
Marine_CP	juvenile	8 (0)	0.68 ± 0.14	40.9 ± 2.94	25.6 ±6.63
Marine_CP	male	5 (0)	2.22 ± 0.31	60.5 ± 3.04	30.2 ±1.48

A total of 120 individuals were measured for SMR, 99 for MMR and 98 for AS (Table S4.2). Sex was a significant predictor of SMR when juveniles were included (lmer: $F_2 = 23.02$, $P < 0.001$) as an additional category, but not when juveniles were removed (lmer: $F_1 = 0.11$, $P = 0.88$). With females as the reference level, males had slightly lower SMR (Estimate =-1.9, SE = 7.76, t = -0.25) and juveniles had higher SMR (Estimate = 90.86, SE = 24.31, t = 3.73; Figure 1a). There was no significant effect of mass (lmer: $F_1 = 7.61$, $P = 0.82$). Ecomorph was a significant predictor of SMR (lmer: $F_2 = 3.76$, $P = 0.023$). With Furnace completely-plated as the reference level, marine completely-plated fish had significantly higher SMR (Estimate = 52.7, SE = 19.88, t = 2.65). This outcome was not affected by the exclusion of juvenile fish (Figure 4.1A). The presence of nuptial coloration in males (n = 31 for presence and n = 27 for absence) was a significant predictor of SMR (lmer: $F_1 = 4.03$, $P = 0.045$), with coloured males having higher SMR than the others. There was no significant effect of mass (lmer: $F_1 = 1.82$, $P = 0.1$). However, the presence of eggs for females (n = 43 for presence and n = 9 for absence)

was not a predictor of SMR (lmer: $F_1 = 0.41$, $P = 0.52$) nor the mass (lmer: $F_1 = 0.066$, $P = 0.94$).

Ecomorph and mass were not significant predictors of MMR (lmer, ecomorph: $F_1 = 0.3$, $P = 0.58$; lmer, mass: $F_1 = 5.37$, $P = 0.29$). However, sex was not a predictor of MMR (lmer: $F_1 = 1.55$, $P = 0.17$) with no significant differences between males and females (Estimate = 47.5, SE = 35.18, t = 1.35) (Figure 4.1B). The presence of nuptial coloration in males (n = 27 for presence and n = 27 for absence) was not a significant predictor of MMR (lmer: $F_1 = 2.7$, $P = 0.10$) but mass was significant (lmer: $F_1 = 5.5$, $P = 0.011$). These predictors are unlikely to be confounded, as coloured and non-coloured males did not differ significantly in mass (Welch's t-test: t = 0.13, df = 48.27, P = 0.90). The presence of eggs for females (n = 39 for presence and n = 6 for absence), was not a significant predictor of MMR (lmer: $F_1 = 0.45$, $P = 0.51$) nor the mass (lmer: $F_1 = 0.23$, $P = 0.48$).

Ecomorph, mass and sex were not significant predictors of AS (lmer; ecomorph: $F_1 = 0.05$, $P = 0.82$; scaled mass: $F_1 = 4.93$, $P = 0.32$; sex: $F_1 = 1.84$, $P = 0.18$) with no significant differences between females and males (Estimate = 50.94, SE = 37.87, t = 1.35; Figure 1c). The presence of nuptial coloration in males (n = 27 for presence and n = 27 for absence) was a significant predictor of an AS (lmer: $F_1 = 4.09$, $P = 0.043$) as well as the mass (lmer: $F_1 = 5.48$, $P = 0.009$), whereas the presence of eggs for females (n = 39 for presence and n = 6 for absence) was not a significant predictor of AS (lmer: $F_1 = 0.20$, $P = 0.65$) nor the mass (lmer: $F_1 = 0.11$, $P = 0.63$). Males showing nuptial coloration had a smaller AS (Estimate = -94.57, SE = 46.76, t = -2.02).

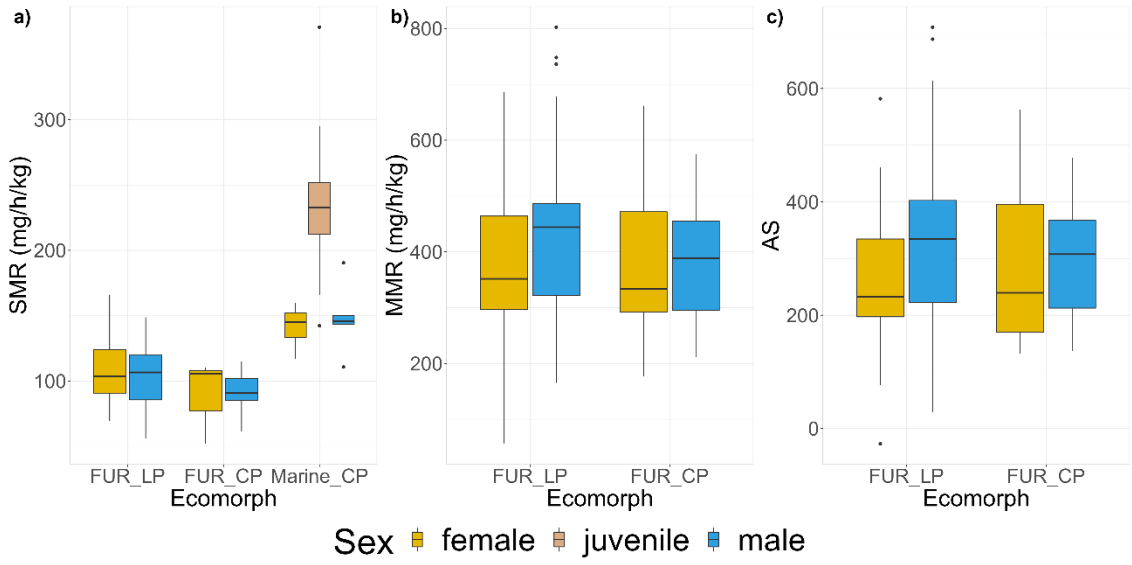

Figure 1. Metabolic rate of the three-spined stickleback for the three different ecomorphs: Furnace low-plated (FUR_LP), Furnace completely-plated (FUR_CP), and Marine completely-plated (Marine_CP) with the different sex category (female, juvenile, male and unknown). a) SMR (mg/h/kg) with n=120; b) MMR (mg/h/kg) n=99; c) AS with n=98.

2. Temperature and salinity

Temperature showed a significant and positive relationship with SMR (lmer: $F_1 = 26.98$, slope = 13.67, SE = 2.63, $P < 0.001$), but did not show any significant relationship with MMR (lmer: $F_1 = 1.18$, slope = 12.48, SE = 11.47, P = 0.27) and AS (lmer: $F_1 = 0.19$, slope = 5.7, SE = 12.87, P = 0.66) (Figure 2. b and d). There was no significant support for any interactions between temperature and sex nor ecomorph.

Salinity showed a significant, positive relationship with both SMR (1) (lmer: $F_1 = 19.1$, slope = 3.1, SE = 0.69, $P < 0.001$) and MMR (lmer: $F_1 = 5.79$, slope = 9.62, SE = 4.0, P = 0.019) (Figure 2. a and c), but no significant relationship with AS (lmer: $F_1 = 2.81$, slope = 7.3, SE = 4.36, P = 0.09). However, when I removed the marine completely-plated group from the SMR analysis, salinity was no longer a significant predictor of SMR (2) (lmer: $F_1 = 0.56$ slope = 0.92, SE = 1.23, P = 0.45), implying that the effect in

the previous model (1) was from the marine fish, but I note that the coefficient is still positive. There was no significant effect of type of day-2 treatment in fish measured twice, and treatment type did not interact with salinity (3), although the slope was again positive (lmer: $F_1 = 0.68$, slope = 1.22, S.E. = 1.47, P = 0.41), supporting the results in (2). However, the addition to the model of a random slope for salinity produced a significant improvement of fit (AIC_C reduction of 6.12). The fixed effect slope of salinity remained positive but non-significant (lmer: $F_1 = 0.95$, slope = 1.49, S.E. = 1.53, P = 0.33), but 73% of individuals would be expected to have a slope > 0. The conditional R^2 of the model with random slopes was 81.8%, implying and extra 8.6% of variance was accounted for by salinity plus its random effect.

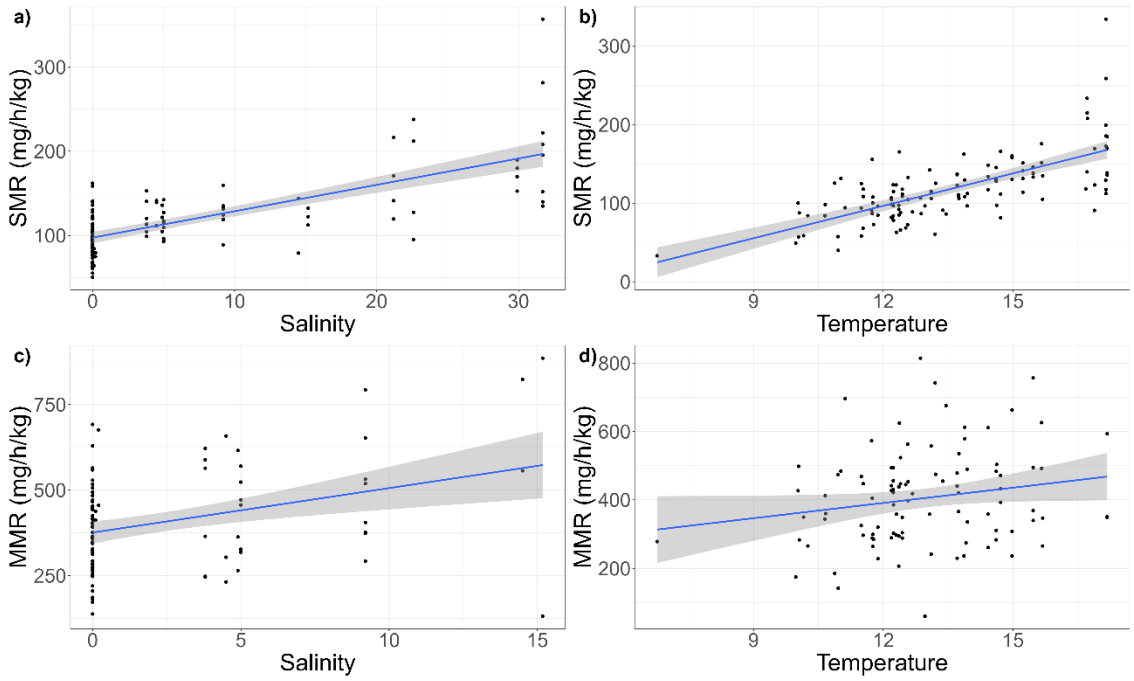

Figure 2. Partial residual plots showing the relationships of SMR (a, b) and MMR (c, d) against salinity (a, c) and temperature (b, d).

3. Recovery time

All comparison of survival analysis models with AIC_C showed that that models with a Weibull error distribution produced better fits than an exponential distribution (Table S4.3). All showed significantly positive rate parameters, in line with the expectation that the probability of an individual reaching its recovery threshold increases with time.

For completely-plated fish from Furnace, five individuals have reached their recovery threshold (median time = 38.5 mins) whereas eight did not. Among low-plated fish from Furnace, 15 individuals reached their recovery threshold (median time = 58.1 mins) whereas 16 did not. No significant differences were found between the Furnace completely-plated (survreg: Z = 0, P = 0.99) and the Furnace low-plated (survreg: Z = 0.63, P = 0.52) in the amount of time required for their recovery from the MMR (Figure 3a).

A total of nine females reached the recovery threshold (median time = 47.1 mins) when ten did not; for males, seven reached the recovery time (median time = 44.7 mins) and 14 did not; all four unknown individuals reached the recovery time (median time = 120 mins). No significant differences in recovery time from MMR were observed among the different sex categories, males (survreg: Z = 0.97, P = 0.33) compared to females (Figure 3b). The scale parameter was 1.47, meaning that the slope of hazard accelerated with time. Similar results were observed when the unknown group was included (survreg males compared to females: Z = 0.93, P = 0.35; survreg unknown compared to females: Z = -1.24, P = 0.25) (Table S4.4). Among males, nuptial coloration (n = 9) was not a significant predictor of recovery time (n = 12) (survreg: Z = -0.87, P = 0.38).

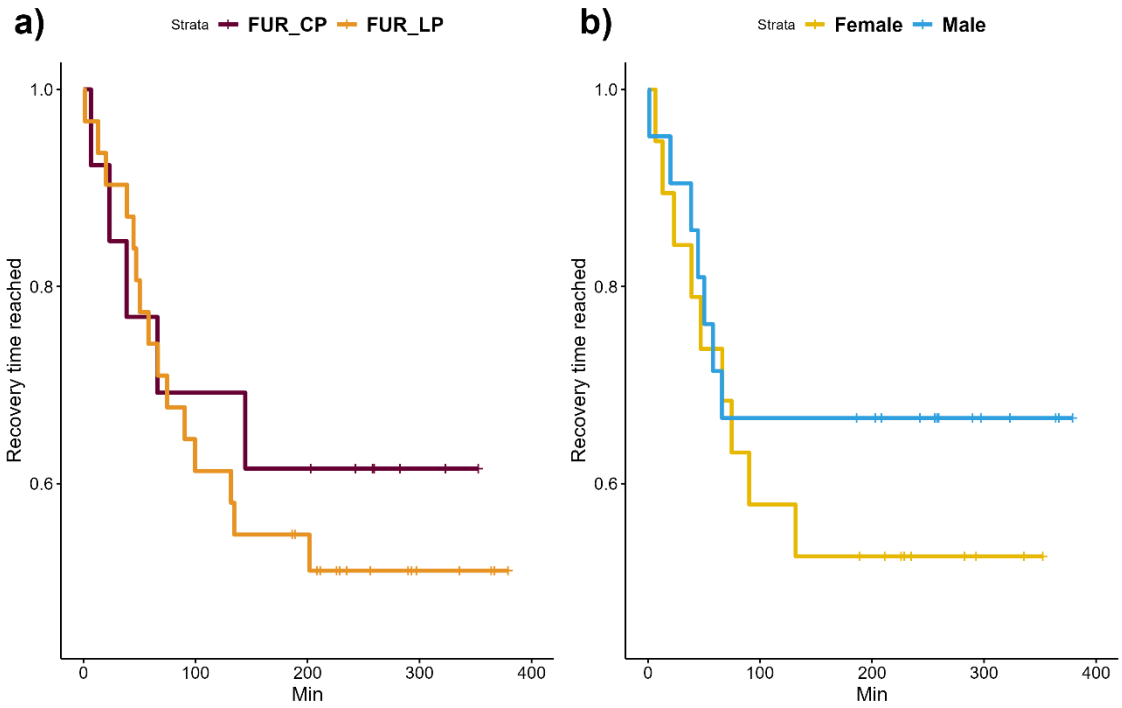

Figure 3. Survival plot of time to recovery threshold: a) for ecomorphs with FUR_CP in purple and FUR_LP in orange; b) for sex with females in yellow and males in blue. Crosses indicate fish that had not reached their recovery threshold by the end of their trial.

Discussion

In my study of metabolic traits in wild sticklebacks, I found that completely-plated fish from a purely marine environment (both adults and juveniles) had a significantly higher SMR compared to completely- and low-plated fish from a brackish environment. Among fish sampled in the brackish environment, completely-plated and low-plated were not significantly different for SMR, MMR and AS. Temperature had a positive relationship only with SMR. Salinity showed a net positive relationship with SMR and MMR, but not AS, though this was hard to separate from ecomorph effects and was complicated by strong between-individual variation in responses to changes in salinity. Males showing nuptial coloration had a higher SMR and a smaller AS than males without coloration, but

no significant difference for MMR. No differences in post-MMR recovery time was observed between completely- and low-plated fish from brackish sites.

Marine fish have significantly higher SMR than brackish-sourced fish

SMR differed significantly according to the marine completely-plated ecomorph (higher than both brackish ecomorphs), juveniles (from the marine completely-plated group, higher than adults from all groups), and male nuptial coloration (higher when coloration present). Within fish sampled from brackish sites (no marine individuals were measured for MMR or AS), MMR and AS differed significantly according to sexual maturity, but only for males. Our finding of marine-sourced individuals having significantly higher SMR than brackish-sourced fish contrasts with previous respirometry work on sticklebacks (Dalziel *et al.*, 2012b), which found no significant difference between marine and freshwater populations. Our results also seem to contrast with previous respirometry work on stickleback salinity regimes (Dalziel *et al.*, 2012b), in that I found marine individuals to have a significantly higher SMR than those from brackish waters, whereas the earlier study found no difference for SMR in marine compared to freshwater populations. However, I cannot rule out that the contrast between studies may arise from different definitions of the ecomorphs. In Dalziel *et al.* (2012), fish labelled 'marine' may be more analogous to our Furnace (i.e. brackish) completely-plated fish, in that they are completely-plated individuals that migrate from the ocean to other environments during the breeding season. Mutual misunderstandings arising from such between-study differences in application of the term 'marine' to sticklebacks have been highlighted elsewhere (Ahnelt, 2018). I take no strong position on choice of terminology, but urge all authors of stickleback research to be aware of the risk of these issues. A similar reason may explain why Dalziel *et al.* (2012) observed a significant difference in both MMR and AS between their marine and freshwater populations, in contrast to our study: I

wascomparing a brackish completely-plated ecomorph with a brackish resident low-plated ecomorph whereas (Dalziel *et al.*, 2012b) compared a marine completely-plated ecomorph with a low-plated freshwater stream resident.

In terms of how differences in metabolic phenotypes traits might relate to the well-characterised morphological differences that often exist between stickleback ecomorphs, general conclusions are hard to draw. In the stickleback system studied by Daniel *et al*., (2012b), parallel work has characterised heritable between-ecomorph differences in several phenotypic traits that may be pertinent to metabolic rates, including body shape, heart size, various components of pectoral fin morphology, and metabolic enzyme activity in muscles (Dalziel & Schulte, 2012; Dalziel *et al.*, 2012a). In Lough Furnace, previous work has found significant morphological and population genetic differences between the ecomorphs (Ravinet, 2012; Ravinet *et al.*, 2015, Leseur *et al.* unpub) and yet the present study found no significant differences in metabolic traits. Results from other teleost families, extrapolated to sticklebacks with due care, may suggest avenues of future enquiry. For example, in Atlantic cod (Gadidae; *Gadus morhua*), higher MMR and greater AS are associated with greater capacity for prolonged swimming (Reidy *et al.*, 2000). In Iberian barbel (*Luciobarbus bocagei)*, fish sourced from a turbulent river had a higher critical swim speed – a method partially analogous to MMR – than fish from a sluggish stream, though this study was not in a position to separate genetic differences from phenotypic plasticity (Alexandre *et al.*, 2014). Perhaps most pertinently, a recent study of the endangered Spanish toothcarp (Cyprinodontidae; *Aphanius iberus*) and an invasive competitor (mosquitofish; Poeciliidae; *Gambusia holbrooki*), both small, euryhaline species, found that toothcarp had a higher MMR and AS than mosquitofish but with a lower swimming performance (Rubio-Gracia *et al.*, 2020). The authors hypothesize that the mosquitofish is able to use a lower proportion of their total aerobic

capacity from swimming compared to the toothcarp. In sticklebacks, it would thus be interesting to measure prolonged swimming performance of our populations to better understand their migratory behaviour, in addition to their use of aerobic capacity for swimming only.

Presence of nuptial coloration has an effect on SMR and AS

The presence of nuptial coloration for males was a significant predictor of SMR and AS, with higher SMR and a smaller AS for males with coloration. However, coloration was not a predictor of MMR, and the presence of eggs in females was not a significant predictor of any measured metabolic trait. While an individual in breeding condition cannot be at its SMR, in the strictest definition of SMR, i.e. not be in a reproductive state (Chabot *et al*., 2016), metabolic assays of the type performed in this study can still provide valuable life-history insights if interpreted with due caution. In the present study, testing for differences in metabolic rates associated with the expression of coloration in reproductive males may provide information on the energetic costs associated with reproduction. The energetic costs to males of producing this coloration are well characterised (Milinski & Bakker, 1990; Candolin, 1999), in addition to the cost of increased predation risk (Johnson & Candolin, 2017) and oxidative damage (Pike *et al.*, 2007; Preston *et al.*, 2011). However, coloration has a positive benefit for males as it represents a strong signalling of their genetic quality to females and hence likely to increase their chance of reproducing (Milinski & Bakker, 1990; Candolin, 1999) as well as other secondary sexual traits such as the quantity of nest threads produced (Östlund-Nilsson, 2001). Such costs would be in keeping with our finding of a higher baseline metabolic rate of coloured males during the breeding season. As there was also no significant difference in MMR associated with male coloration, males may be using up part of their aerobic scope in the production and maintenance of colour and its associated

costs even though males with no nuptial colours were also sexually mature (by observation of the gonads). A recent study has shown that secondary sexual traits such as courtship quality metrics, aggression to other males and red nuptial coloration were not related to SMR or MMR, but that males with a nuptial coloration had a higher level of oxidative damage in the sperm DNA (Chiara *et al.*, 2022). The contrast between that study's results and mine is hard to account for as I only measure the presence or absence of coloration for males. To resolve this, future work would need to quantify coloration differences or other secondary sexual traits like nest building, courtship behaviour (Chiara et al., 2022) or threads quantity (Östlund-Nilsson, 2001). For females, the absence of significant SMR and MMR differences between gravid and non-gravid can be explained by our small sample size of non-gravid females and also the fact that females can have multiple clutches during one breeding season (Wootton, 1984), and it was difficult to assess if non-gravid females had yet to begin egg development, were between clutches, or were fully spent.

Salinity has a positive effect on SMR and MMR with a strong between-individual variation

My results showed that higher salinities were associated with significantly higher SMR and MMR, but there was no significant association with AS. However, when I removed the marine individuals, the effect, though still positive, was no longer significant, implying that this effect was being largely driven by the marine completely-plated group. Further support for a lack of strong, consistent effect of salinity within the brackish-sourced fish comes from restricting the dataset to the fish measured twice as part of the salinity change experiment, though the slope was again positive. However, this analysis also indicated significant between-individual variation in the response to salinity, suggesting that salinity is, indeed, an important predictor of stickleback SMR, but that its

influence is subject to a certain amount of context dependency. Future work to unpick this relationship would involve increasing sample sizes, increasing the range of salinity changes to which experimental fish are subject to, and, if possible, testing individuals at more than two salinities.

As a general rule, teleost fish tend to have their lowest metabolic rates in the salinity of their normal habitat, though this is by no means universal, (Ern *et al.*, 2014). However, in my study, in which individuals were only exposed to salinities within the range they would typically experience, higher salinity was associated with higher SMR. Moreover, because I manipulated salinity on a subset of individuals, a certain amount of causality likely applies to this relationship. That marine completely-plated fish had the highest SMR cannot be attributed to their being completely plated, as there was no significant difference between brackish-sourced fish of contrasting plate phenotype. Even if my results did not show a significant effect of salinity on SMR, I observed that salinity only affects some individuals rather than all of them. Therefore, my result agrees with a previous stickleback respirometry studies that found no differences in SMR associated with native salinity regime or plate phenotype, in addition to no differences associated with plating phenotype, and that salinity treatments did not produce a change in SMR. However, there are many differences in the methods between the earlier study and mine. In Grøtan *et al*., (2012), all fish were acclimated to one of several constant salinities for two weeks before respirometry, whereas I assayed fish 24 hrs after capture, restricting our salinity ranges to those the fish would typically experience. The time of sampling could also be responsible for these differences as they sampled in august, which is the end of the breeding season, whereas my sampling took place at different time of the year, but most individuals were captured during the breeding season. The absence of strong differences between the two different salinity treatments can be related to the fact that,

some studies have found no differences in osmoregulation energy demand by measuring the gill Na+K+-ATPase activity between freshwater and marine populations that were both tested in 0 or 30 PSU (Jurss *et al.*, 1982). Similar results have been observed between one freshwater and two brackish populations from the Baltic Sea when they were exposed to 0 or 20 PSU (Schaarschmidt *et al.*, 1999). Conversely, other studies have suggested that there may be osmoregulatory ability differences between sticklebacks from different origins and populations (e.g., Tudorache *et al.*, 2007; Kitano *et al.*, 2010). In particular, gene expression work using artificially-crossed within- and between-ecomorph families reared under freshwater and high-brackish common-garden conditions showed differential gene expression of candidate osmoregulatory genes in gill tissue (McCairns & Bernatchez, 2009). The study also highlights potential phenotypic plasticity in stickleback osmoregulation and discusses the possibility that freshwater lineages have lost some of this plasticity, though this interpretation is caveated by the potential for artificial rearing to have influenced the investigated traits. It may thus be that changes to energy expenditure associated with osmoregulation can only be detected in extreme and/or abrupt changes. However, my results argue for some degree of short-term plasticity in SMR, as experimental reductions in salinity within a naturally-experienced range resulted in lower SMR values. Collectively, these previous results and my highlight the complexity of the interplay between osmoregulation and energetics in individual's life-history and species.

Temperature has an effect on SMR but does not affect AS

The positive relationship I observed between temperature and SMR is in agreement with other studies that have shown the same pattern. In previous work, sticklebacks from a freshwater environment that experience a potential temperature variation from 4 to 21°C were acclimated to different temperature treatments (5°C, 12°C and 20°C) and presented

a significantly lower SMR and AS at 5°C compared to the other treatments (Ressel *et al.*, 2021) with a linear increase of SMR when temperatures increase. However, SMR and AS did not differ significantly between 12°C and 20°C treatments. In this same study, MMR was also significantly lower when the temperature was lower but only between the two extreme treatments (5°C and 20°C) while 12°C treatment was a midpoint between the other, but no significant differences were observed with 5°C and 20°C. My findings broadly reflect this, though the positive relationship was not significant. In Iceland, stickleback populations residing in a naturally warm lake (15-20°C) had a lower SMR compared to the populations that were residing in a colder environment (10-15°C) when they were acclimated and kept at the same temperature treatment (10°C, 15°C and 20°C) (Pilakouta *et al.*, 2020). This study also found evidence to support the idea that an increase in temperatures causes a greater increase in SMR than in MMR, implying that the increase in respiration rate may be 'eating into' a fish's AS (Donelson *et al.*, 2011). This is analogous to the effect I observed for male nuptial coloration. In the nine-spined sticklebacks (*Pungitius pungitius*), freshwater and marine populations were caught in the wild and acclimated to different temperature treatments in a common garden (6°C, 11°C and 19°C) (Bruneaux *et al.*, 2014). They showed that freshwater and colder populations have a better thermal compensation (which is a greater SMR than MMR, resulting in the decline of AS at different temperatures outside the optimal window) compared to the others. My results showed that higher temperature affect SMR but not MMR nor AS, which could suggest that energy is being used in compensation to high temperature, even if results are not significant for AS.

Ecomorph and sex do not affect the recovery time after exhaustion

Ecomorph and sex were not significant predictors of recovery time. These results are in disagreement with previous work which showed that low-plated individuals

outperformed completely-plated individuals in fast-start events, but that the relative performances (maximum and mean velocities, greater distance travelled) were reversed in prolonged swimming trials. In fast-start experiences, low-plated sticklebacks have been found to have better performance (maximum and mean velocities, greater distance travelled) than completely-plated sticklebacks, whereas completely-plated were better at prolonged swimming exercise (Taylor & McPhail, 1986; Bergstrom, 2002). Indeed, due to their different life history traits, completely-plated individuals present different migratory behaviours (like anadromy or partial anadromy) that require different physical and physiological capacities (Kitano *et al.*, 2012), while low-plated sticklebacks are usually resident in brackish or freshwater environment. Differences in predation pressure have been proposed as a driver of the loss of lateral plates in lake- and stream-resident populations (Reimchen, 2000). For low-plated sticklebacks, a fast-start escape response is crucial to escape a predator whereas the completely-plated individuals are protected by the bony armour plates. Under such a model, I might expect in our study that low-plated fish would recover slower from exhaustion as they show higher response to fast-start challenge (Bergstrom, 2002) following the vigilance-driven hypothesis (Millidine *et al.*, 2006; Killen *et al.*, 2015). I did not observe any differences in time recovery between coloured males and the others, even if coloured males have a significantly higher AS and that individuals with higher AS tend to have longer recovery time (Killen *et al.*, 2015). Nevertheless, my results did not show any significant differences between coloured and uncoloured males in MMR, suggesting that all males expend similar levels of energy during exercise and do not differ into recovery. Measuring additional traits linked to secondary sexual traits like the difference of coloration intensity could also be a good proxy to better assess how reproductive status affects the metabolic rates of males.

Methodological caveats for comparisons between respirometry studies

To estimate the 'true' SMR, fish should not be in a reproductive state (Chabot *et al.*, 2016). However, assessing metabolic traits during breeding periods is still of ecological and evolutionary pertinence, and species-specific constraints may render breeding seasons the only time at which individuals may be catchable. In my system, the breeding season was the only time of the year when purely marine sticklebacks were present in the rock pools (Ross Finlay pers. comm. and pers. obs.), and when the brackish completely-plated individuals in Lough Furnace are sufficiently abundant. Similarly, sticklebacks in Lough Furnace are less active and less catchable during winter – the ideal study period when juveniles would be large enough to assay and not yet reproductive. A certain amount of care is also needed when comparing MMR results between studies, both in absolute values and statistical significance, as debate exists around the relative merits of different methods, including as to how well any of these reflect a conceptually 'true' MMR (Norin & Clark, 2016). In their stickleback study, (Ressel et al., 2021) observed that chasing, a version of which was also deployed here in my study, did not consistently induce swimming (escape) behaviour. In my study, I was satisfied that I was capturing the pertinent biology with a robust proxy, as most fish responded well to chasing and had reliably reached a tiredness plateau by two minutes. Although my SMR range values were similar to Ressel *et al*., (2021), my MMR range values were somewhat lower: 250-750 mg/h/kg, compared to their 300-1200 mg/h/kg. There are too many differences between our protocol and theirs for us to feel unduly troubled by this relatively modest difference. As examples to explain these differences, it could be that all of their fish were kept in an almost complete freshwater conditions (0.35 PSU), the fact that all their fish were captured in september, and that respirometry trials were done 42-60 weeks after their original capture. Fish were kept for a long time under similar conditions of temperature, food and light exposure. In to Ressel *et al*., (2021), there is also no

information about individual's sex or reproductive state. Therefore, all of these could contribute to the differences I observed between studies.

My study shows that different ecomorphs (low- and completely-plated) residing in the same brackish environment have no major systematic differences in their metabolic phenotypes, compared to a purely marine resident and compared to each other. While females and males did not differ significantly in their metabolic rates, being in breeding condition as indicated by nuptial coloration may cost males a portion of their aerobic scope. The key abiotic factors of temperature and salinity may affect SMR but do not seem to affect AS. Furthermore, no factor was identified to reach the potential recovery time, and no differences between ecomorphs or sex were observed. Collectively, the nuances of these results, and their various agreements and disagreements with previous work on this species, may ultimately underscore the complexity of stickleback evolutionary history, a history that makes it at once both a first-class model species and a species about which is itself difficult to generalise.

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Supplementary material

Table S4.1. Summary of the main sampling location and different sites with their different parameters: Lat : latitude, Long : longitude, Total : total number of fish caught at this site, Min : minimum salinity measured at this site, Max : maximum salinity measured at this site, mean : salinity mean calculated from all the salinity measured, Median : median salinity of all salinity measured at this site.

Location	Site	Lat	Long	Total	Min	Max	Mean	Median
FUR	NB	53.91	-9.57	37	2.6	28.5	9.54	7.4
FUR	Shed	53.90	-9.58	30	4.7	18.5	6.5	5
FUR	Slo	53.92	-9.58	41	0.2	18.6	8.8	5.7
FUR	SP	53.91	-9.58	14	0.8	13	6.1	4.9
MU	RP	54.10	-9.99	20	16.2	35.9	23	23.6

Table S4.2. Summary of the number of fish per group for the different metabolic rates: SMR, MMR and AS; Ecomorphs: FUR_CP = completely-plated form Lough Furnace, FUR_LP = low-plated from Lough Furnace, Marine_CP = completely-plated fish from Muingdoran.

	SMR			MMR		AS	
Ecomorph	FUR_CP	FUR_LP	Marine_CP	FUR_CP	FUR_LP	FUR_CP	FUR_LP
female	7	40	5	8	37	7	37
juvenile	0	0	10	12	42	12	42
male	12	41	5	0	16	0	16

Table S4.3. Summary of the different models tested with different variables and parameters with their associated AICc.

Model	AICc
Model 1 :Ecomorph_Weibull	281.23
Model 1_b :Ecomorph_Exponential	283.63
Model 2 : Sex_Weibull	279.23
Model 2_B : Sex_Exponential	280.84
Model 2_c : Sex_Weibull (unknow removed)	225.43

Table S4.4. Summary of the model output.

	Value	Std.Error	z	p
(Intercept)	6.082	0.52	11.71	<2e-16
Sexmale	0.727	0.748	0.97	0.331
SexUNK	-1.115	0.9	-1.24	0.216
Log(scale)	0.384	0.206	1.86	0.063

980

Chapter 5 : Intraspecific variation and environmental predictors shape gut microbial communities in wild caught three-spined sticklebacks (*Gasterosteus aculeatus*)

Abstract

Genetic and environmental drivers of gut microbiome are difficult to disentangle in the wild. Looking at within-species variation, especially when associated with genetic and ecological specialization of hosts, can provide insights into the factors that drive gut microbiome communities and their functional potentials. In this study, I compare gut microbiome communities of ecomorphs of the three-spined stickleback (*Gasterosteus aculeatus*) in the Burrishoole system at the coast of western Ireland. In this system, morphology and population genetics distinguish four ecomorphs: 1) a low-plated freshwater resident; 2) a low-plated brackish resident; 3) a completely-plated, brackish, putatively anadromous; and 4) a purely marine, completely-plated ecomorphs. Water samples were collected as environmental controls. Alpha diversity of gut microbial communities differed significantly by both ecomorph and sex. Community composition differed significantly between host (midgut) and environment (water) for marine fish, but not for other ecomorphs. The proportions shaping the microbial community composition at the phylum level were similar across the fish gut and in environmental samples, with *Proteobacteria* and *Firmicutes* making up the largest proportions. The results of a distance-based Redundancy Analysis showed that the number of lateral plates and

environmental salinity were the strongest predictors of beta diversity. These results suggest that biotic factors (e.g., diet) and abiotic (e.g., salinity) might be key drivers of microbiome composition and diversity. Identifying which specific factors shape co-evolved bacteria within their host could lead to understand the potential adaptive role of microbiome.

Keywords: Microbiome, ecomorph, stickleback

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Introduction

Commensal and symbiotic microbiome communities play a key role in the ecology, physiology and evolution of animals (Turnbaugh *et al.*, 2006; Ley *et al.*, 2008; Tremaroli & Bäckhed, 2012; McFall-Ngai *et al.*, 2013). The composition of microbial communities can impact host performance and fitness (Gould *et al.*, 2018; Walters *et al.*, 2020), and its potential role in evolution continues to gain attention (McFall-Ngai *et al.*, 2013; Rudman *et al.*, 2019). Correlations between host phylogenetic distance and microbiome community divergence are regularly reported (Ley *et al.*, 2008; Ochman *et al.*, 2010; Brooks *et al.*, 2017; Kohl *et al.*, 2018), and may contribute to reproductive isolation and speciation (Rudman *et al.*, 2019). Therefore, studying the interactions between host and microbial communities is essential to evaluate the relative importance of genetics, environment, and gene-by-environment interactions in shaping gut microbiome composition.

Microbial communities present in the gut of an individual are abundant, diverse, and impact host health, development and immune system function (Balcázar *et al.*, 2007; Cheesman *et al.*, 2011; Lathrop *et al.*, 2011; Zhu *et al.*, 2011). Although between-species variation in gut microbial community composition is typically greater than that within species, microbial diversity and abundance can also vary dramatically between individuals of the same species or population. Numerous case studies have shown that microbial composition can vary according to host genotype (Rawls *et al.*, 2006; Benson *et al.*, 2010; Kovacs *et al.*, 2011), rearing environment, and contemporary surrounding environment (De Filippo *et al.*, 2010; Muegge *et al.*, 2011; Wu *et al.*, 2011; Koeth *et al.*, 2013). The environment affects components of the external microbial community that can colonize the gut through food intake and digestion, while features of the host

environment and genotype filter community member establishment (Ley *et al.*, 2008; Benson *et al.*, 2010; Kovacs *et al.*, 2011). Relatively few studies have focused on understanding the migration rate of the bacterial communities from the environment (the source) to the gut, or on determining which communities shape the stable core (i.e., common groups of microbes or genes that are likely to be very important for host biological function) and which are transient (Cheaib *et al.*, 2020; Heys *et al.*, 2020).

The major environmental factor that shapes the vertebrate gut microbiome is diet (Ley *et al*., 2008; David *et al.*, 2014; Delsuc *et al*., 2014; Smith *et al*., 2015). Diet serves both as a source of colonization (Costello *et al*., 2012) and as an abiotic influence on the gut environment that microbes colonise (David *et al*., 2014). However, within natural populations or closely related species, individuals living in the same environment may specialize in different diets, and may also differ in degree of specialization (Bolnick *et al.*, 2003; Araújo *et al.*, 2007). For example, in the swallowtail butterfly *Papilio machaon*, subspecies often utilise contrasting host plants, while the closely-related sister species *P. zelicaon* (formerly considered a subspecies) is polyphagous (Thompson, 1998). Within the same population, individual European oystercatchers (*Haematopus ostralegus*) often show contrasting dietary preferences and feeding strategies (Goss-Custard & Durell, 1983) that may expose them to different environmental microbiota sources. In aquatic systems, another important environmental predictor is salinity (Herlemann *et al*., 2011; Logares *et al*., 2013). A study of water samples collected of 2,000 km of the Baltic Sea observed spatial shifts in the microbial community structure from the estuary to the open sea (Herlemann *et al.*, 2011), with a decline in the number of observed sequences matching "marine" sequences when the salinity decreases, but an increase the number of sequences matching "freshwater" sequences (a bacterial example

of the so-called 'Remane relationship' (Remane, 1934) of the richness of freshwater and marine species along a salinity gradient).

In addition to environmental factors, bacterial composition can also differ between ecotypes within species (Sullam *et al.*, 2015; Sevellec *et al.*, 2018). In Trinidadian populations of guppies (*Poecilia reticulata*), two stream-resident ecotypes, one from a high-predation site and the other from a low-predation site, were reared under the same lab conditions and presented significantly divergent microbiome communities, suggesting that host genetics affects microbiome competition (Sullam *et al.*, 2015), although the comparison of only two sources means that non-selective, deme effects cannot be ruled out. Similar results were observed for two ecotypes of the lake whitefish (*Coregonus clupeaformis*) (Sevellec *et al.*, 2018). This study found that the dwarf limnetic and the normal benthic ecotype microbiome communities differed not only from their environment but also from each other. Even though the ecomorphs typically shared 44% of the core microbiome (ranging from 22-65% of shared genera), the authors also suggested that community composition was influenced by host physiology, immunity and genetic background. These studies demonstrate the complexity of the interaction between host and microbiome communities. Sex also plays an important role in influencing the composition of the host gut (Freire *et al.*, 2011; Kovacs *et al.*, 2011; Costello *et al.*, 2012; Koren *et al.*, 2012; Bolnick *et al.*, 2014c; McGee & Huttenhower, 2021). For example in mice, host gender driven gut microbial composition is well-characterised and has been observed in numerous studies on a wide range of animal taxa (Bolnick *et al.*, 2014c), though the majority of the differences remain individual-specific (Org *et al.*, 2016; Kozik *et al.*, 2017). A recent study on wild meerkats (*Suricata suricatta*) found that even circadian rhythms affect the gut composition, eclipsing seasonal and age effects (Risely *et al.*, 2021). These drastic variations could potentially

be explained by the temperature fluctuations in an arid climate zone, as meerkat foraging behaviour is based on temperature. However, it underscores the importance of individual biology and metabolism in shaping gut microbiome.

The three-spined stickleback (*Gasterosteus aculaetus,* L. 1758; henceforth just 'stickleback') is a small teleost fish distributed across the temperate and subarctic areas in the Northern Hemisphere (Wootton, 1984), with a long history as a model species in ecology and evolutionary biology (Ostlund-Nilsson *et al.*, 2006; Huntingford & Ruiz-Gomez, 2009; Barber & Nettleship, 2010). Sticklebacks have independently and repeatedly colonized freshwater environments approximately 11,000 years ago, after the last glacial maximum. This colonisation has led to repeated adaptation to brackish and freshwater conditions, a phenotypic variation, and multiple divergent ecomorphs (Bell & Foster, 1994). A phenotypic trait of particular interest has been the partial or complete loss of lateral bony plates in freshwater populations (Bell *et al.*, 2004; Barrett *et al.*, 2008). Three main different ecomorphs have been described based on the number of lateral plates: (Münzing, 1963; Hagen & Gilbertson, 1973): completely-plated, observed in marine and brackish environments and potentially anadromous; partially-plated, found in brackish environments; and low-plated, found in freshwater and brackish environments.

Sticklebacks reside in and can tolerate a broad range of salinity, from purely freshwater to purely marine (Wootton, 2009). Several studies on stickleback gut microbiome have shown strong influences of diet and a strong difference between the sexes (Bolnick *et al.*, 2014c). Another study showed the major histocompatibility complex gene family – a critical component of the adaptive immune response in vertebrates – affected the gut microbiome in natural populations of stickleback (Bolnick *et al.*, 2014a). More recent work has shown that gut microbial communities have shifted repeatedly in the same

direction in association with parallel divergence and speciation among different stickleback ecomorphs (benthic-limnetic and marine-freshwater) (Rennison *et al.*, 2019), highlighting the importance of the ecomorphs but also of the life-history of sticklebacks.

This study aims to investigate within- and between-ecomorph variation in stickleback gut microbiome composition and diversity. The four different ecomorphs were characterised as follows: 1) a low-plated freshwater resident; 2) a low-plated brackish resident; 3) a completely-plated, brackish, putatively anadromous; and 4) a purely marine, completely-plated ecomorphs. I sequenced the V1 hypervariable region of the 16S rRNA gene of gut and water bacteria to test the differences between gut and water bacteria. Ultimately, our goal was to determine the relative roles of genetics and environment in the diverse composition of the fish gut microbiome in naturally caught fish.

Methods

1. Study system

Sticklebacks were sampled in two main locations in the west of Ireland: the Burrishoole catchment (N53°55′22″, W9°34′20″), and Muingdoran (N54°10′30″, W9°99′04″), located 50 km north from the Burrishoole. The Burrishoole catchment is a network of rivers and lakes connected to the Atlantic Ocean via the estuary Clew Bay. The two main lakes are Lough Feeagh (410 ha) and Lough Furnace (141 ha) (Poole & de Eyto, 2006; Cassina, 2012). Lough Feeagh is an oligotrophic freshwater lake with a maximum depth of 46 m (de Eyto *et al.*, 2016). Feeagh is connected to Furnace by two channels: the natural Salmon Leap and the artificial Mill Race. Lough Furnace is a 20 m deep and permanently stratified tidal lagoon (Cassina, 2012; Cassina *et al.*, 2013), with stable stratified salinity layers between the surface and deeper water maintained by a strong and

shallow pycnocline (~2.4 m), high concentrations of dissolved oxygen in the surface, but anoxic conditions below ~6-7 m depth (Kelly *et al.*, 2018a,b). In the surface layer, Lough Furnace displays a strong north-south salinity gradient with low concentrations in the north part of the lake (from 2 to 4 PSU) to brackish concentrations in the channel that connects to Clew bay (10 to 15 PSU). In comparison, Lough Feeagh is purely freshwater (0 PSU), is only stratified in summer, and is strongly influenced by rainfalls and floods. This bay embraces a high habitat diversity and is an Area of Conservation (NPWS, 2015). In Muingdoran, fish were sampled in purely marine rock pools along the shoreline, directly connected to the Atlantic Ocean at high tides and but often disconnected during low tides.

2. Sample collection and preparation

From may 2020, unbaited minnow traps (silver galvanized Gee-type model #1279 Frabill, Jackson, Wisconsin, USA) were set at three different sites across Lough Furnace and two sites at Lough Feeagh (Figure 5.1). Traps were also set in several rock pools in Muingdoran, all considered as one site. Traps were set from 1-2 m of the shoreline, at 0.5-2.5 m depth in Lough Furnace and Lough Feeagh whereas traps were set in the middle of the rock pools (depth <1 m) at Muingdoran. Traps were set out early in the morning and collected 48 hours later to cover two tides (with two high tides and two low tides). Salinity and temperature were measured at each site when collecting traps (WTW Multi 340i Handheld Multimeters, Germany).

In total, 93 three-spined sticklebacks were collected (S5.1). All fish were brought to the laboratory within 1-2 hrs of capture and euthanized with 1.0 g/L of MS-222 solution (tricaine methanesulfonate, FVG Ireland). Only adults (sexually mature individuals) were used. Sex was determined by visual observation of the gonads during dissection

(S1), which also confirmed sexual maturity. A small bias in favour of females was observed and was likely due to the different territorial behaviour between the sexes during the breeding season, as males stay closer to nests and are thus slightly less mobile than females.

The number of lateral plates was counted under a binocular magnifier (Stereo Binocular, Euromex, magnification 0.7-4.5×) using tweezers. Total length was measured to the nearest mm using a calliper, weight to the nearest 0.1 g. As the gut length was not recorded, I used the total length as a proxy for the gut length, as it was showed in mammals and herbivores (Clauss *et al.*, 2007; Lavin *et al.*, 2008), that the gut size is proportional to animal's size. A small fin clip (ca. 2 mm^2) was taken and preserved in 1 mL absolute ethanol.

Fish were dissected under aseptic conditions maintained under a Bunsen flame. The entire gut tract was extracted, placed in a sterile tube, flash-frozen on dry ice, and preserved at -80 °C.

Water samples were collected at each time and location of fish capture (S1). Water was sampled in 1.5 L plastic bottles disinfected with 10% bleach before sampling, and rinsed 10 times with site water prior to sample collection. Samples were preserved at 4°C and filtered on the day of collection. Water was filtered through a 0.22 µm filter (MF-Millipore MCE membrane, USA) via a peristaltic pump (Masterflex model 7518-00), under aseptic conditions and a Bunsen flame. Between 500 mL to 700 mL were filtered per sample, depending on the amount of suspended elements saturating the filter. Filters were stored in sterile tubes (Cryotubes ClearLine), flash-frozen on dry ice, and stored at -80°C. The filtering system was bleached between each sample. To test for contamination of the tubing and filtration system, routine negative controls (during the water filtration)

were regularly taken, after bleaching and rinsing the system with autoclaved water (S5.2).

After rinsing, I used autoclaved water on filters to check for any contamination.

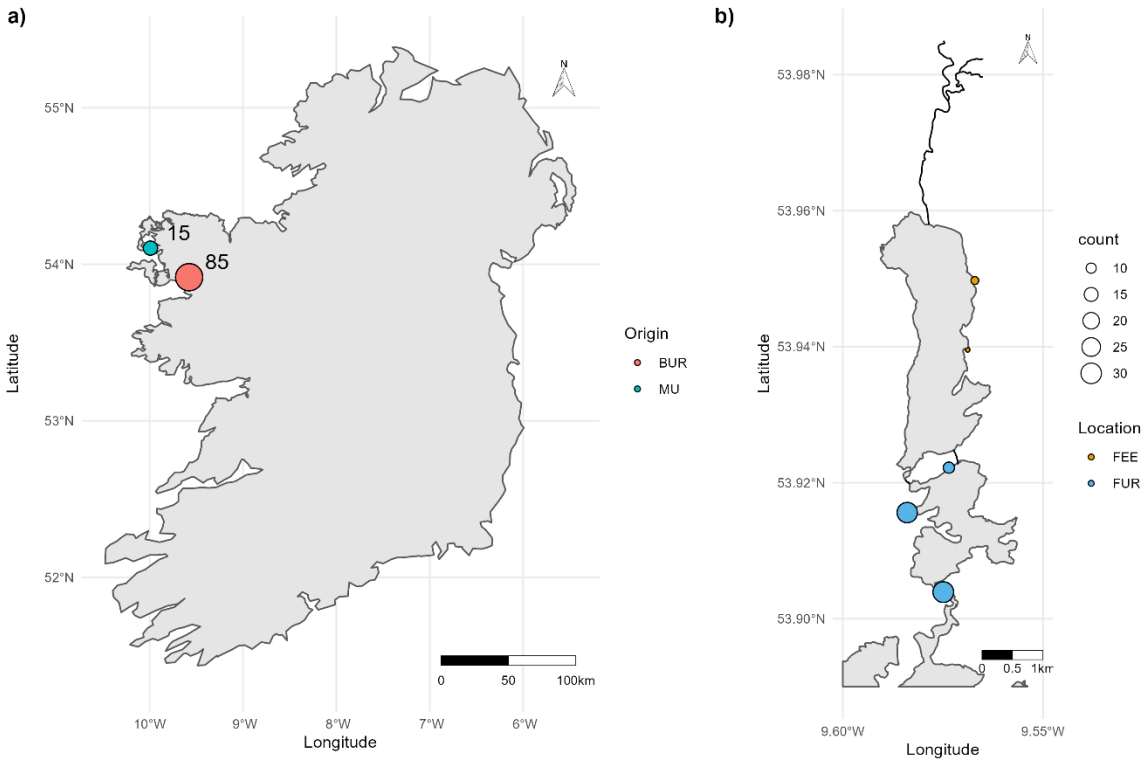

Figure 5.1. a) Map of Ireland showing the two main locations on the west coast of Ireland: Muingdoran (blue) and Burrishoole (red) with the number of fish caught per location; b) The Burrishoole system with the two main locations: Lough Feeagh (orange) and Lough Furnace (blue). The circle size is proportional to the number of fish caught at a given site.

3. DNA extraction, amplification and sequencing of 16S rRNA genes

For samples from fish, DNA was extracted from a small portion of the foremost anterior part of the gut, including content (~3 g). For water samples, filters were cut and crushed into small pieces before extraction under aseptic conditions. I similarly processed one

randomly-selected negative control from the beginning, middle and end of the sampling period to test for contamination.

DNA extraction was undertaken using the QIAamp Stool kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, with minor adjustments described in the supplementary material (S5.3).

I amplified and sequenced the V1 hypervariable region of the universal rDNA 16S gene. PCR conditions and individual barcoding are as Heys *et al.* 2020. To reduce the impact of the 'jackpot effect' (the possibility of a polymerase lesion being indistinguishable from a variant mutation in the DNA fragment) and chimaeras (a sequence containing sequences from different amplicons), three PCR replicates were performed for each sample and the PCR products pooled. Post-PCR DNA quantity was determined on an agarose gel, after which samples were purified using magnetic beads for each sample (0.7× times). As none of the negative samples showed evidence of amplification on an agarose gel, they were not sent for sequencing. DNA concentration of each sample was assessed using a Qubit Fluorometer (Life Technologies, 2015). After quantification, the libraries were equimolarly pooled together (20 ng of amplicon per sample). The quantity and quality of DNA in the final library were assessed on a gel in triplicate. The pooled library was sequenced on an Illumina NovaSeq PE250 platform (250 bp paired-end reads).

4. Bioinformatics and OTU analysis

Sequence analysis was performed using the bioinformatics pipeline described in Heys *et al.* (2020). Briefly, I trimmed the sequences with the software SICKLE v 1.2 (>Q27 for

quality score) (Joshi and Fass 2011). Read error correction was performed with the software SPAdes v2.5.0 to obtain good quality of forward and reserve assembly (Nikolenko & Alekseyev, 2011; Bankevich *et al*., 2012). I then merged paired-end reads (overlap length of 70 bp) using the software PANDAseq v2.11 with the "simple Bayesian read-merging" algorithm (Masella *et al*., 2012). Merged paired-end reads were de-replicated, sorted and filtered from singletons, and chimeras using the software VSEARCH v2.3.4 (Rognes *et al*., 2016). In fact, two chimera removal were performed the first was a de novo removal followed by a reference-based removal using GOLD SILVA reference dataset. Afterwards, the decontamination from host sequences were performed by mapping reads against stickleback genome using the bwa algorithm implemented in the tool Deconseq (standalone v 0.4.3). Merged pair-end filtered, chimeras-free and decontaminated reads were clustered into Operational Taxonomic Units (OTUs) with the algorithm "usearch_global" implemented in the software VSEARCH, specifying 97% identity. The taxonomic assignment for OTUs was performed with Naïve Bayesian Classifier (Pedregosa *et al*., 2011) implemented in QIIME2 using the SILVA database (Bolyen *et al*., 2019). Multiple sequence alignment was performed with MAFFT (Katoh & Standley, 2013), and phylogenetic trees of OTUs were generated using the software FastTree (Price *et al*., 2010). The OTU dataset was then converted into a biological observation matrix (BIOM) format to predict the function categories and metabolic pathways using Tax4Fun software (Aßhauer *et al.*, 2015).

5. Post-OTUs statistical analysis

All subsequent analyses were performed in the statistical software R v4.1.0 (R Core Team, 2018). Species richness and effective Shannon index were used as metrics of alpha diversity and were calculated using the *Rhea* package (Lagkouvardos *et al.*, 2017). I used

linear mixed models with the lmer function from the *lme4* package (Bates *et al.*, 2015) to test for effects of ecomorph, sex and total length on each of the richness indices, using sampling site as a random effect. Total length was scaled within each ecomorph. I further confirmed the strength of the linear mixed model with a Kruskal-Wallis test, which was used to assess whether either metric of alpha diversity differed significantly according to group (including between gut samples and water samples). While not strictly necessary given that our data met the parametric assumptions for the linear mixed model, such non-parametric tests, typically less powerful than their parametric analogues, are often used in microbiota analyses owing to frequent departures from parametric assumptions. The computed P values from these tests were corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate method (Benjamini *et al.*, 1995).

Beta diversity (dissimilarity in community composition between samples) was assessed using generalised UniFrac (Chen *et al.*, 2012), which accounts for both species abundance and evenness in the calculation of the phylogenetic distances.

Differences in community structure between groups (ecomorphs or sex) were analysed using a distance-based redundancy analysis (dbRDA) approach, with permutational multivariate analysis of variance (PERMANOVA) used to assess statistical significance.

To test for significant differences in functional profiles (predictions of metabolic functions for bacteria) between groups, I used a Kruskal-Wallis test, following up any statistical significance with pairwise Mann-Whitney tests corrected for multiple comparisons (Benjamini *et al.*, 1995).

Results

1. The effects of ecomorph and sex on alpha diversity

Ecomorph and sex were both significant predictors of the effective richness (lmer, plate type: $F_{1,3} = 7.8$, $P < 0.001$; sex: $F_{1,1} = 4.1$, $P = 0.043$), but there was no significant interaction between them (lmer: $F_{1,3} = 0.1$, $P = 0.96$). For the second model, ecomorph was a significant predictor of the effect richness (lmer, ecomorph: $F_{1,3} = 4.6$, $P = 0.0031$). However, total length (lmer, length: $F_{1,1} = 11.43$, $P = 0.46$), sex (lmer: $F_{1,1} = 2.48$, $P = 0.12$) and the interaction variables were not significant (lmer, length:ecomorph : $F_{1,3} = 0.23$, $P = 0.91$; length:sex: $F_{1,1} = 0.03$, $P = 0.87$). Ecomorph was a significant predictor of the Shannon effective index richness (lmer: $F_{1,3} = 7.7$, $P < 0.001$), whereas sex and the interaction between ecomorph and sex were not significant (lmer: sex: $F_{1,1} = 0.16$, $P = 0.68$; sex: $F_{1,3} = 0.24$, $P = 0.86$). The second model with the total length included, only the ecomorph was a significant predictor of the Shannon effective (lmer: $F_{1,3} = 7.4$, $P < 0.001$), while sex (lmer: $F_{1,1} = 0.85$, $P = 0.33$), total length (lmer: $F_{1,1} = 2.39$, $P = 0.15$), and the two interactions were not significant (lmer, length:ecomorph : $F_{1,3} = 0.46$, $P = 0.79$; length:sex: $F_{1,1} = 0.045$, $P = 0.83$).

For the effective richness comparisons, marine environmental samples were significantly different from those of both Lough Furnace and Lough Feeagh ($P = 0.012$ and $P < 0.01$ respectively) with a lower effective richness, while environment samples from Lough Feeagh and Lough Furnace were not significantly different ($P = 0.3$). For marine samples, fish guts were significantly different from their respective environmental samples ($P =$

0.02), while fish guts from Lough Feeagh were not significantly different from their environment samples (P = 0.567). Completely-plated and low plated fish from Lough Furnace were not significantly different from their environment (P = 0.35 and P = 0.16 respectively).

For the effective richness comparisons, marine environmental samples were significantly different from those of both Lough Furnace and Lough Feeagh (P = 0.02 and P < 0.01 respectively) with a lower effective richness, while environment samples from Lough Feeagh and Lough Furnace were not significantly different (P= 0.54). For both marine and Feeagh samples, fish guts were significantly different from their respective environmental samples (P = 0.023 and P = 0.042 respectively) (Table 5.1). However, fish from Lough Furnace, both low-plated and completely-plated, were not significantly different from their environment (P = 0.16 and P = 0.38 respectively). All pairwise comparisons of gut samples between fish populations showed significant differences (P < 0.018), except for Furnace low-plated v. Furnace completely-plated (P = 0.79).

For the Shannon effective pairwise comparisons, marine environmental samples were significantly different from those of Lough Feeagh (P < 0.01), but neither of the other two pairwise comparisons between environmental samples showed a significant difference (Furnace vs Feeagh: P = 0.39; Furnace vs marine: P = 0.20). Fish gut samples were all significantly different compared to their originated environment samples (P < 0.01), except for the marine fish samples that were not significantly different from marine environment (P = 0.56) (Table 5.1). For pairwise comparisons of gut samples between fish populations, marine individuals were significantly different from all the other groups (P < 0.018). However, low-plated from Lough Furnace were not significantly different from completely-plated Lough Furnace (P = 0.95) nor from low-plated from Lough

Feeagh (P = 0.11). Low-plated individuals from Lough Feeagh were also not significantly different from completely-plated from Lough Furnace (P = 0.22).

Table 5.1. Summary of significant pairwise differences, after correction for multiple comparisons, between treatment groups ordered from the highest difference to the smallest for each metric of alpha diversity.

Index	Group 1	Direction	Group 2	Comparison
Effective Richness	Marine	>	LP_Fe	fish vs fish
Effective Richness	Marine	>	LP_Fur	fish vs fish
Effective Richness	LP_Fur	>	LP_Fe	fish vs fish
Effective Richness	Marine	>	CP_Fur	fish vs fish
Effective Richness	CP_Fur	>	LP_Fe	fish vs fish
Effective Richness	Marine	>	Env_Mar	fish vs env
Effective Richness	Env_Fe	>	LP_Fe	fish vs env
Shannon effective	Env_Fe	>	LP_Fe	fish vs env
Shannon effective	Env_Fur	>	LP_Fur	fish vs env
Shannon effective	Env_Fur	>	CP_Fur	fish vs env
Shannon effective	Marine	>	CP_Fur	fish vs fish
Shannon effective	Marine	>	LP_Fur	fish vs fish
Shannon effective	Marine	>	LP_Fe	fish vs fish

2. The effects of ecomorph and sex on beta diversity

The number of lateral plates and site salinity were both significant predictors of weighted UniFrac distance between pairs of samples when they were taken separately (P < 0.001) or taken together (P = 0.004 and P < 0.001 respectively). Moreover, when removing the marine individuals, these predictors were still significant when taken separately (P < 0.001 and P = 0.011 respectively) or together (P = 0.008 and P = 0.011). By using the weighted UniFrac distance again, the only non-significant comparisons between ecomorphs were Marine vs LP_Fur (P = 0.075) and CP_Fur vs LP_Fur P = 0.081). When compared using Bray-Curtis distance, only CP_Fur vs LP_Fur was not statistically

significantly (P = 0.076). Sex was not a significant predictor of weighted UniFRac distance between samples, taken the three variables together (P = 0.33) or separately (P = 0.33).

Results of distance-based redundancy analysis (dbRDA) showed that the number of plates and site salinity influence the beta diversity differences between groups, with the first axis, mainly affected by salinity, explaining 81.32% of the explained variance and the second axis, mainly driven by the number of plates, explained 18.68% of the variance (Figure 5.2). When rerun without the marine individuals, the first axis was mainly driven by the number of plates and explained 74.77% of the variance, while the second axis was mainly driven by salinity and explained 25.23% of the variance.

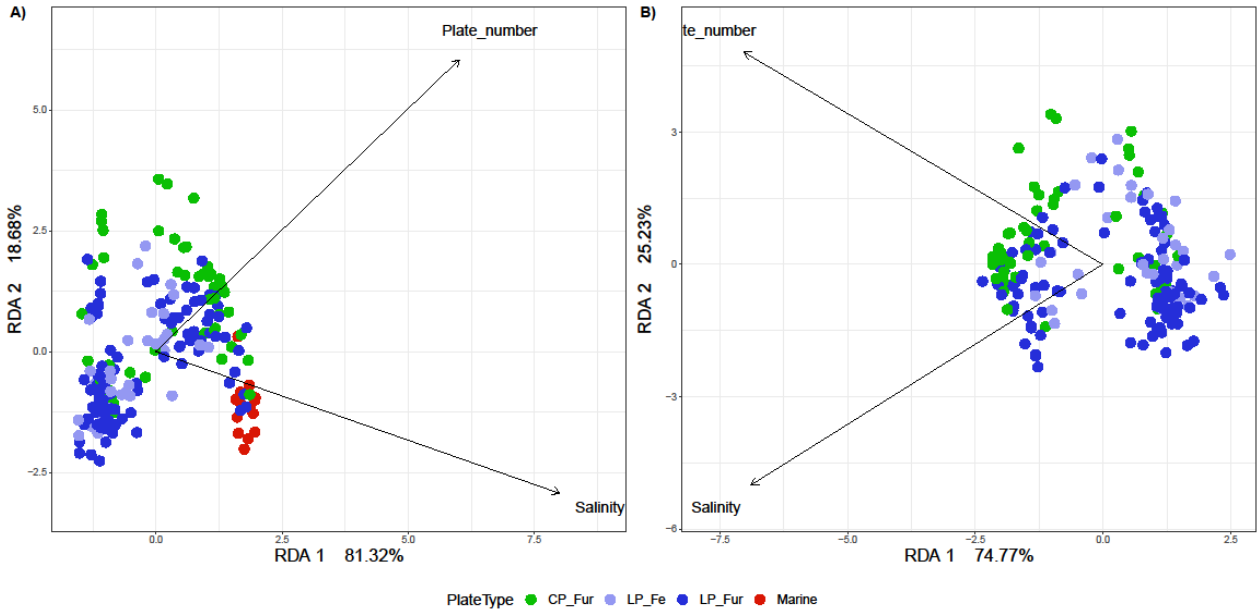

Figure 5.2. Distance-based redundancy analysis (dbDRA) with the percentage of variation explained on each axis: a) with the four ecomorphs, b) without the marine ecomorph. Ecomorphs are the following: CP_Fur = completely-plated from Lough Furnace, LP_Fe = low-plated from Lough Feeagh, LP_Fur = low-plated from Lough Furnace, Marine = completely-plated from Muingdoran.

3. Taxa abundance and OTUs shared between fish gut, fish gut and their environment, and between environment samples

At the phylum level, the most abundant group was *Proteobacteria* in all gut and environmental samples (~50-60%; Figure 5.3). *Firmicutes*, *Actinobacteria* and *Tenericutes* were present at lower but constant proportions in fish and environmental samples. The proportions for the most abundant phyla were similar across the different groups.

I observed that 22.1% of OTUs were shared between low-plated Feeagh fish gut and their environment, while 64.6% were only in the fish gut (Figure 5.4). For the marine group, only 0.2% of OTUs were shared between the fish gut and their environment and 88% were found in the fish gut only. For Lough Furnace, both low-plated and completely-plated fish shared 4.8% of OTUs with their environment while they share 65% of OTUs. They each shared a small proportion of OTUs with their environment but not with each other (0.4% for completely-plated and 2.4% for low-plated).

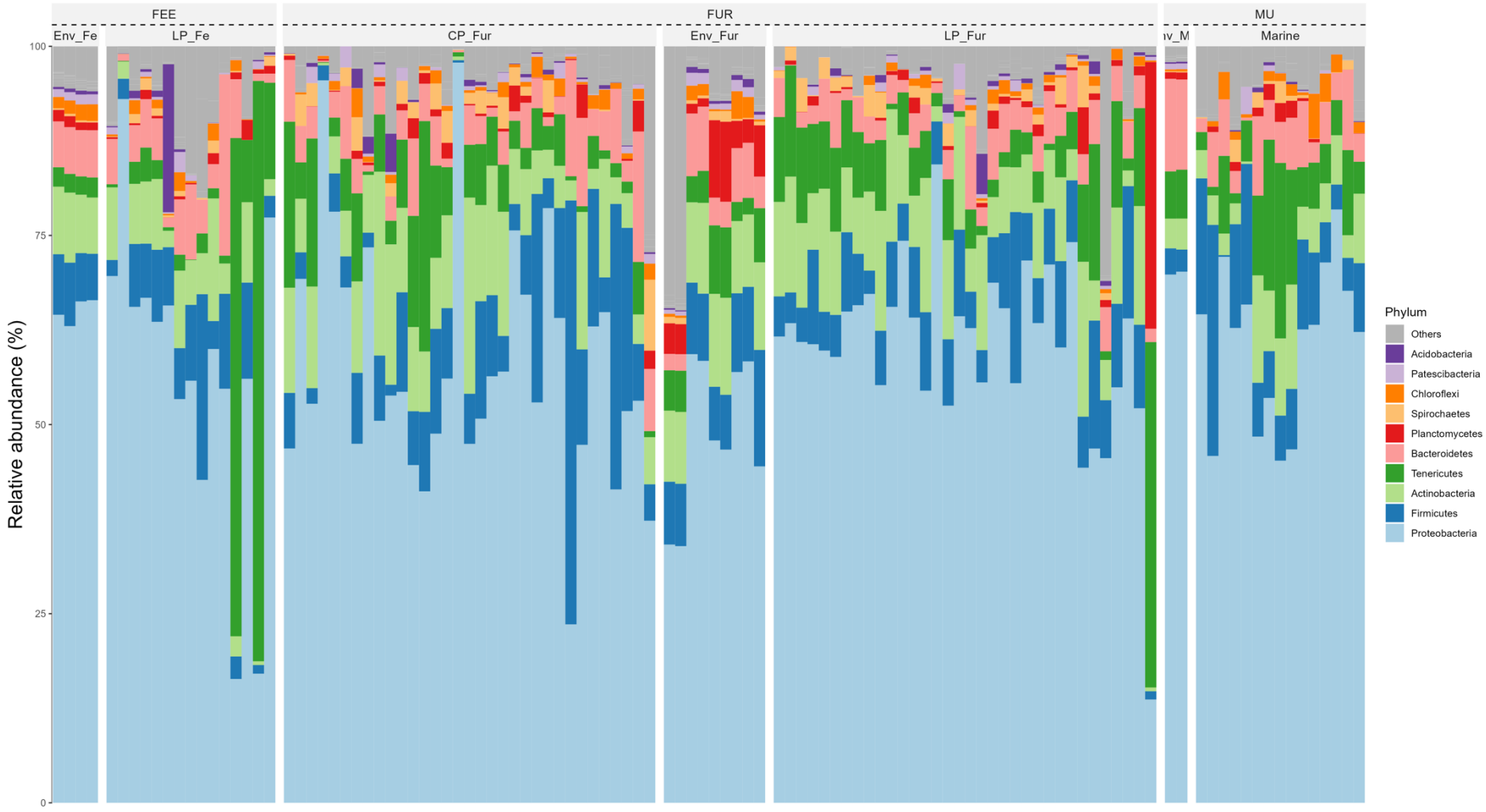

Figure 5.3. Percentage of the relative abundance of the taxa at the phylum level per group (environmental samples and ecomorphs). Ecomorphs

are the following: CP_Fur = completely-plated from Lough Furnace, LP_Fe = low-plated from Lough Feeagh, LP_Fur = low-plated from Lough Furnace, Marine = completely-plated from Muingdoran. Environment samples: Env_Fe = water samples from Lough Feeagh, Env_Fur = water samples from Lough Furnace, Env_Mar = water samples from Mugdoran.

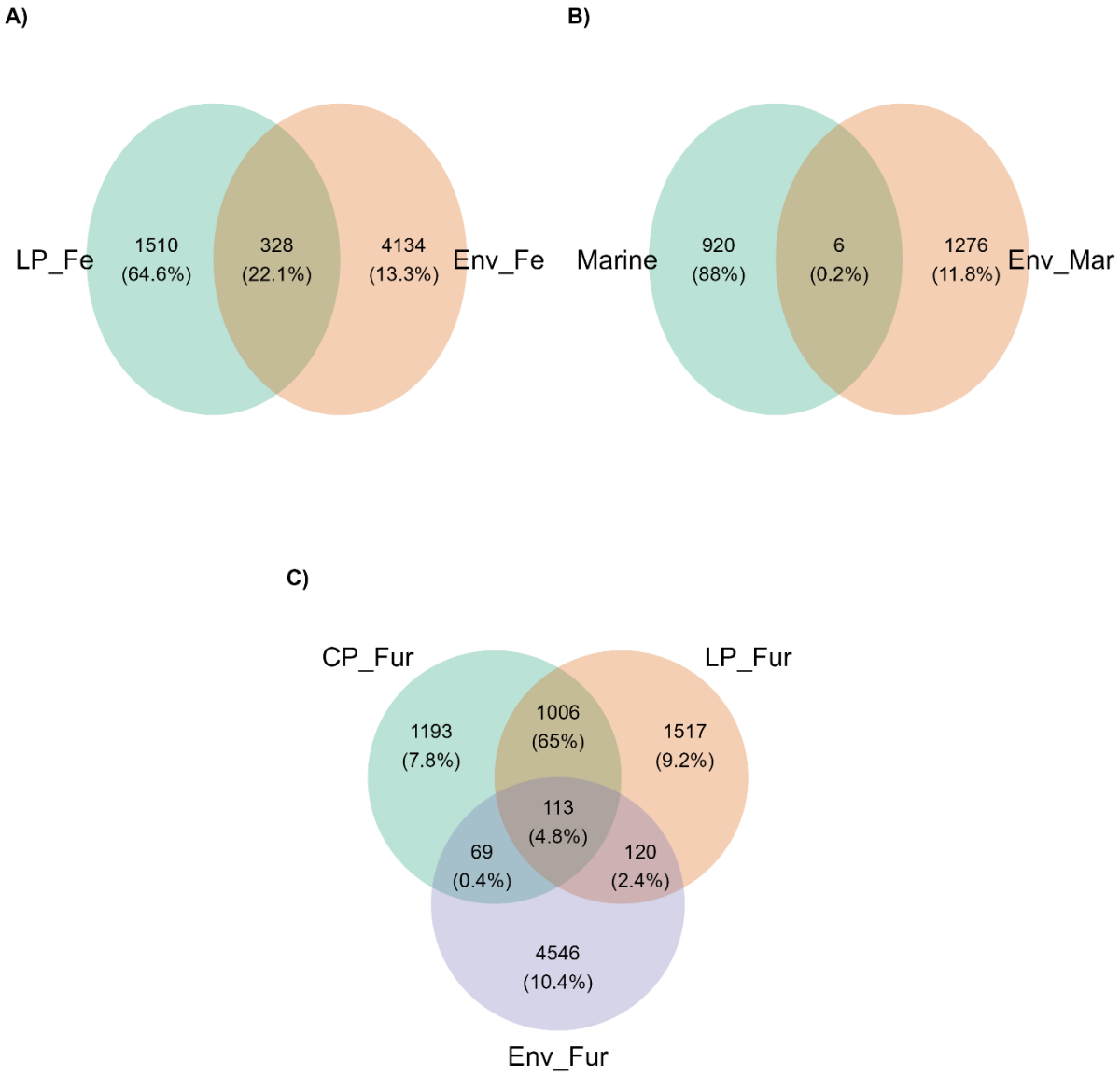

Figure 5.4. Venn diagrams representing OTUs: a) Lough Feeagh fish versus Feeagh environmental samples; b) Marine fish vs marine environmental samples; and c) Lough Furnace fish of both plating classes versus each other and versus Furnace environmental samples.

4. The effect of ecomorph on functional diversity

Several metabolic pathways showed significant pairwise differences. Of these, the comparison of Lough Feeagh and Marine fish had the highest number of pathways showing significant differences (n = 28), and the lowest being between low-plated fish

from Lough Furnace and completely-plated Lough Furnace (n = 6; Table 2). More generally, the Marine group had the highest significant differences from other groups in functional content.

These differences in the function the microbial communities have were linked to different super classes of functions such as carbohydrate biosynthesis or degradation, amino acid biosynthesis, and fatty acid and lipid biosynthesis.

Table 2. Summary of the number of different functional abundance comparisons between two groups. HP: comparison number, GR1: group 1, GR 2: group 2 and DIF: number of functional profiles showing significant differences in abundance on 491 functions tested.

HP	GR1	GR2	DIF
1	LP_FE	LP_FU	14
2	LP_FE	CP_FU	11
3	LP_FE	Marine	29
4	CP_FU	LP_FU	6
5	LP_FU	Marine	18
6	CP_FU	Marine	19

Discussion

I found significant predictors of gut microbial community composition and diversity in the three-spined stickleback. Species richness was mainly predicted by ecomorph and sex whereas diversity was mainly predicted by ecomorph. For each ecomorph, gut microbial richness or diversity differed from the respective environment, and only the marine group differed from its environment for both richness and diversity. The main predictors of these differences were the number of lateral plates and the salinity from the sampling site. The number of lateral plates and, therefore, ecomorph, was the strongest driver of the variation within or between host populations. I also observed significant

differences in functional microbial community composition between ecomorphs, with the lowest number of differences between low-plated and completely-plated within Lough Furnace, and the highest number of differences between low-plated from Lough Feeagh and marine.

Species composition of gut microbiota is known to be strongly modulated by host diet (Zhu *et al.*, 2011; Sullam *et al.*, 2012; Bolnick *et al.*, 2014c; David *et al.*, 2014). In our study, I did not directly assess the diet for each ecomorph. However, Ravinet (2012) showed, based on a stomach content analysis, that the different ecomorphs residing in the Burrishoole catchment were feeding on different types of prey items. Low-plated fish from Lough Feeagh were mainly feeding on freshwater prey (85%, the rest being made up of food items possibly of terrestrial origin), whereas low-plated fish from Lough Furnace were feeding on both freshwater (34%) and marine (38%) prey items. Completely-plated fish in Lough Furnace showed a stronger marine bias (62% marine, 10% freshwater). Assuming that these descriptions of diet are representative of sticklebacks in the Burrishoole, our results agree with previous stickleback microbiota work showing a relationship between diet and community traits, (e.g. Bolnick *et al.*, 2014c). Besides the diet, gut size can also influence the diversity of microbiome, as a longer gut (more volume) can host more microbial communities and in turn, the communities can shape the morphogenesis (gut size) (Gordon & Bruckner-Kardoss, 1961; Sommer & Bäckhed, 2013; Huang *et al.*, 2018). However, the food quality can directly affect the gut size, more specifically a higher quality diet results in smaller gut size in grouse, primates and fish (Moss, 1972; Chivers & Hladik, 1980; Sullam *et al.*, 2015a). In this study, the gut length was not available, and the total length of the fish was used as a proxy for the gut length. The total length of the fish was centred within each ecomorph to control for the fact that completely-plated individuals were longer than the

low-plated. As total length was not a significant predictor of the richness and the composition, longer individuals within each ecomorph did not have a higher composition or richness in communities compared to the smaller one. As diet was not observed in this study, no strong conclusion can be made. However, a previous study has showed that diet differed significantly in composition among each ecomorph (Ravinet, 2012), but did not give information about the food quality nor if there was variation within the same ecomorph. Assumptions can be made that if the fish size is a good proxy for gut size, fish within ecomorph have similar quality diet.

Previous work has shown salinity to be a predictor of microbial diversity in the water (Logares *et al.*, 2009; Herlemann *et al.*, 2011) and in fish gut (Schmidt *et al.*, 2015). In water samples along a salinity gradient in the Baltic Sea, there was a gradual transition from "freshwater" sequences to "marine" as salinity increased (Herlemann *et al.*, 2011). Even if I could not test for such transition in our system (as our sample size for environmental samples was too low, especially for the rock pools n = 2), I suspect that salinity is shaping the differences in microbial communities in the environmental samples. For example, in Atlantic Salmon (*Salmo salar*), gut microbial community assemblage differed significantly by life-cycle stage, especially in marine adults, which showed enriched diversity within common marine taxa (Llewellyn *et al.*, 2016). Our study supports this: even when the marine group was dropped, salinity in the Burrishoole system still explained 16% of the variance on the first axis of the dbRDA, while salinity explained 20% when the marine individuals were included. In Mexican Mollies (*Poecilia sphenops*), individuals from a common starting salinity (~1 ppt) were acclimated to one of 0, 5, 18 or 30 ppt salinity treatments (Schmidt *et al.*, 2015). While gut microbiota communities at the end of the experiment were only weakly correlated with either the initial or final environmental communities, there was a complete turnover of OTUs

between 0 ppt and 30 ppt, with the transition already evident in the 5 ppt group. This suggests that even minor increases in salinity can change host microbiome, and in ways not easily predicted by the microbial composition of the surrounding water. They also, however, suggest that there might be functional redundancy between distinct communities, whereby a change in taxonomic profile does not automatically mean a change in functional profile. Such redundancy has previously been suggested to influence the assembly of host (Burke *et al.*, 2011) and environmental communities (Frossard *et al.*, 2012). Our results broadly agree with these three studies, in that salinity is a key predictor of the gut microbial community. An important caveat to (Schmidt *et al.*, 2015) is that they used the entire fish rather than just the gut, meaning their analysis reflects the pooled microbial communities from the skin and other tissues, in addition to the gut. However, that caveat arguably underscores their argument that the microbial community of the water is having minimal impact on the microbial community of the host fish, as either the skin microbiome is experiencing minimal influence from the water microbiome, or that any influence on the skin is not strong enough to overcome the lack of influence on other microbiota components.

In several studies, sex has been shown to influence the gut microbiome (Freire *et al.*, 2011; Kovacs *et al.*, 2011; Costello *et al.*, 2012; Koren *et al.*, 2012; Bolnick *et al.*, 2014c; McGee & Huttenhower, 2021). A meta-analysis examined gut microbiota data from humans, laboratory mice, two wild fish species (stickleback and Eurasian perch) and one laboratory fish (stickleback), and found a strong genotype-by-environment interaction effect on the microbial composition, in which females and males differed in their response to diet (Bolnick *et al.*, 2014c). For instance, in sticklebacks, female microbiome responded more strongly to diet than males. Intriguingly, when assessing the effect of diet on stickleback (from Vancouver Island, Canada) and European perch (*Perca*

fluviatilis, from Erken Lake, Sweden), the two hosts presented similar sex-specific impact of diet, with similar difference in females vs males (Bolnick *et al.*, 2014b). Surprisingly in our study, sex was not a strong predictor of microbial diversity, even though our sample size was well balanced between females and males. While this result argues against a strong effect of sex in this system, it is also possible that our sample size is too low to allow any sex effect to come through after controlling for other predictors.

In our study, I found that microbial diversity and composition differed significantly between ecotypes, with the strongest contrasts involving the marine group. However, I did not find any significant differences in the microbial diversity and richness between low-plated and completely-plated ecomorphs from Lough Furnace. Moreover, the number of plates also predicted the differences between the different ecomorphs. A caveat is that the number of lateral plates and salinity are partially confounded, as predictors are slightly correlated (Pearson test, cor = 0.33, P <0.001). Of the two, however, the number of lateral plates remains the stronger predictor (86% of variance explained with the marine individuals and 46% of variance explained without the marine individuals, vs 20% for the salinity with marine individuals and 16% without marine individuals). This result is consistent with previous studies on other fish taxa that have observed ecomorph-based microbial community divergence (Sullam *et al.*, 2015; Sevellec *et al.*, 2018). In those studies, ecomorphs were residing in contrasting habitats and predation regimes (guppies, *Poecilia reticulata*) (Sullam *et al.*, 2015), or had contrasting limnetic and benthic life histories (lake whitefish, *Coregonus clupeaformis*) (Sevellec *et al.*, 2018). However, most ecomorph studies have focused on freshwater pairs (limnetic-benthic axis), and relatively few studies have observed freshwater-marine pairs. A study of two different limnetic-benthic species pairs of Midas cichlid (*Amphilophus zaliosus* and A. *sagittae* for the limnetic pairs, *A. astorquii* and *A.*

Amarillo, for the benthic pairs), residing in two different saline lakes, found that the ecomorphs of one of these two pairs presented significantly different microbiome communities even though they were captured as juveniles in the Apoyo lake and reared under similar laboratory conditions (Franchini *et al.*, 2014). This argues for a stronger influence of genotype over the rearing environment in shaping lifetime microbiota. However, the two other ecomorphs from the Xiloá lake did not have significantly different microbial communities. The authors suggest that this might be a consequence of the lake age, as Xiloá lake is younger (~6,100 years) than Apoyo lake (2,4000 years), and therefore, the ecomorphs differentiation is more recent in Xiloá. They also found that the differences between limnetic and benthic fish from the two lakes were lower compared to the differences between ecomorphs within the same lake, i.e. between-ecomorph difference was lower than between-lake difference. Despite these differences, they found that the most common community was members of the *Oceanospirillales* order, reported as halotolerant bacterial communities.

In my system, I found that all ecomorphs were significantly different from each other, except the completely-plated v. the low-plated within Lough Furnace. In population genetic terms, low-plated stickleback residing in Lough Feeagh are probably the most isolated of our sampled sites in the Burrishoole, as immigration from Lough Furnace, if it does occur, is likely exceptionally rare (Ravinet *et al.*, 2015) (Chapter 5). In contrast, Lough Furnace is a secondary contact zone between a low-plated resident ecomorph and a completely-plated, potentially anadromous ecomorph, and also receives a low level of immigration from Feeagh. Hybridisation between completely-plated and low-plated seems to be low but existent, at least in one direction (Chapter 2, Chapter 3). Given a certain amount of time in a common environment coupled with a potential for gene flow between LP and CP, albeit limited, it is perhaps not surprising in Lough Furnace that LP

and CP fish do not significantly differ in their microbial communities. More recently, a study of independently-evolved benthic and limnetic stickleback pairs showed consistent parallel changes in the composition and function of the gut microbiome (Rennison *et al.*, 2019). Parallelism across the different ecomorph pairs for functional microbial diversity was more pronounced than taxonomic composition diversity, suggesting that divergence may influence their performance under similar environmental constraints. In our study, the greatest number of significant comparisons involving divergence in the 'metabolism' and 'biosynthesis' functional groups were between low-plated from Lough Feeagh and marine individuals, followed by both completely-plated and low-plated ecomorphs from Lough Furnace to marine. This pattern is congruent with other phenotypic and genetic differences observed in the Burrishoole (Chapters 2, 3 and 4). The lowest number of significantly different functional pathways was between low-plated and completely-plated ecomorphs from Lough Furnace. Therefore, location seems to be the strongest factor influencing the gut microbiome composition and function in these populations.

Extensive previous work has shown the important influences of host genotype on gut microbial communities' assemblage. Furthermore, numerous studies, including meta-analyses, have shown that host genotype and host diet are arguably the strongest predictors of community similarity/dissimilarity (Roeselers *et al.*, 2011; Sullam *et al.*, 2012). While I were not able to directly test diet effect in this study, I would recommend analysing the microbiome of the potential prey present in the environment that stickleback might feed on and also, compare it to separate analyses of gut content and gut tissue, to disentangle what community are found in the stickleback food and the differences in the resident communities between the different ecomorphs. It would also be informative to investigate the potential differences these ecomorphs have for genes associated with the immune system (major histocompatibility complex, MHC).

Moreover, many other factors that I did not investigate in this study can modulate the assemblage of microbial communities, such as parasite infection (Hahn *et al.*, 2022), time of the day (Risely *et al.*, 2021) or the migration (Risely *et al.*, 2018). It would also be interesting to further investigate the level of parasite prevalence for stickleback by *Schistocephalus solidus* within this system, though it would take many years and/or intensive sampling to build up a sufficient sample size (around 50 out of 1,110 individuals sampled, Leseur, personal observations).

Our study shows that ecomorphs from three different locations (purely freshwater, brackish lagoon and purely marine) have significantly different microbial compositions and that they present differences in their functional pathways. However, the low-plated and completely-plated ecomorphs living in the brackish lagoon do not present significant differences in their composition and have the lowest number of functional differences. Both the number of the plate and the salinity from the residing environment play a key role in modulating the microbial composition of the different ecomorphs. Sex did not seem to play a major role in shaping the communities in these ecomorphs, compared to other studies. Collectively, these results show that the major factors to influence the gut microbial composition and function are both biotic and abiotic sources. Therefore, these results bring some insights into the potential role that microbiome communities could play in reproductive isolation between the allopatric and sympatric ecomorphs, while abiotic factors seem to play an important role between sympatric ecomorphs.

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Supplementary material

Table S5.1. Summary of the main sampling location and different sites with their different parameters: n_env = number of environmental samples, n_female = number of sampled low-plated females and completely-plated in brackets, n_male = number of sampled low-plated males and completely-plated in brackets, Location = lake of origin, Date = day of sampling, Lat : latitude, Long : longitude, Sal_avg = the average of all the salinity recorded this sites, Sal_min = the minimum salinity recorded at this site, Sal_max = maximum salinity recorded at this site.

Site	n_env	n_female	n_male	Location	Date	Lat	Long	Sal_avg	Sal_min	Sal_max
TH	2	4	4	Feeagh	21/05/2020	53.95	-9.57	0	0	0
EB	2	5	2	Feeagh	22/05/2020	53.94	-9.57	0	0	0
MI	2	5 (2)	3 (1)	Furnace	18/05/2020	53.92	-9.57	1.8	1.1	2.6
Slo	2	7 (7)	7 (8)	Furnace	19/05/2020	53.92	-9.58	5.9	2.3	11.2
7A	5	7 (8)	8 (7)	Furnace	24/05/2020	53.90	-9.57	10.8	3.9	18.9
RP	2	10	5	Muingdoran	18/05/2020	54.10	-9.99	31.7	22.6	35.9

Table S5.2. Summary of the environmental controls and their parameters: n_cont = number of samples, n_DNA_amp = number of processed samples (DNA extraction and amplification), Amplification = control of amplification after PCR on agarose gel, Location = lake of origin, Date = date of sampling.

Site	n_cont	n_DNA_amp	Amplification	Location	Date
TH	2	1	No	Feeagh	21/05/2020
EB	2	0	NA	Feeagh	22/05/2020
MI	2	1	No	Furnace	18/05/2020
Slo	2	1	No	Furnace	19/05/2020
7A	2	0	NA	Furnace	24/05/2020
RP	2	1	No	Muingdoran	18/05/2020

S5.3. DNA extraction protocol modifications from QIAamp Fast DNA Stool Mini Kit

1. Prepare a 1cm2 cut of Whatman qualitative filter paper No. 1 in sterile condition
2. Deposit a drop of collected faecal material on the paper
3. Let air dry for approx. 5 min at room temperature
4. The piece can now be stored in -80◦C for long preservation or directly used to continue the extraction
5. Transfer each cut into a separate 1.5 ml Eppendorf and label with sample name
6. Add with 100 µl of FTA Purification Reagent
7. Incubate 5 min toom temperature
8. Remove the reagent and rinse with 200 µl of TE buffer
9. Remove carefully all TE buffer
10. Add 14 µl of Solution 1
11. Incubate at room temperature for 5 mins
12. Add 26 µl of Solution 2 to each Eppendorf
13. Incubate room temperature for 10 mins
14. Shake or votex for 10 sec
15. Centrifuge for 20 sec at 2000 rpm
16. Transfer 40 µl in a new sterile Eppendorf consisting in your final eluted DNA

Chapter 6 : General discussion

The Burrishoole ecosystem complex located in western Ireland comprises a sequence of headwater rivers and freshwater and tidally influenced lake components, eventually leading to the open sea. Lough Furnace, a brackish water lagoon within the complex, is considered to be an important secondary contact zone (a secondary contact zone is formed between two populations that have diverged by genetic drift or selection during a period (or periods) of isolation prior to contact) offering opportunities for the comparative study of the biology and factors mediating the divergence of co-occurring populations of marine and freshwater types stickleback (Ravinet *et al*. 2015). The objective of this study was to further characterise morphologically (Chapter 2), genetically (Chapter 3), physiologically (Chapter 4), and microbiologically (Chapter 5), intraspecific variation among the stickleback found in the Burrishoole ecosystem complex. Based on a combination of new data acquired in the project, knowledge of the physical geography and different environmental attribute, like salinity, and insights derived from the interpretation of combined neutral and non-neutral variation in this and the previous study, it was possible to reflect on how that variation and associated processes might have developed and be maintained. Also, the degree to which processes producing the intraspecific variation observed within the Burrishoole ecosystem complex might parallel processes resulting in similarities in the nature of intraspecific variation found in other marine-freshwater transition contact zones could be considered. I found substantial genetic variation revealed by a combination of 30 neutral and adaptive markers and evidence that population divergence has likely resulted from a combination of evolutionary influences (a scenario of isolation-by-environment for neutral markers, where the genetic distance increases drastically with an abrupt environmental change; a

scenario of isolation-by-adaptation for the adaptive markers, as the genetic distance increases with a phenotype change). There was also only sparse indication that the population diversity observed in Burrishoole and that their genetic underpinnings parallelism with what had been observed in other freshwater-marine transition areas.

A synthesis of new findings and insights

There was sufficient morphological evidence based on a reanalysis of lateral plates number to support the presence only of two ecomorph populations in Lough Furnace instead of the three, including a partially-plated form, originally proposed in a previous study by Ravinet *et al*. (2015). With a substantial level of coherence between the morphological assignation of population and the genetic allocation of population groups, I was able to use this combination of genetic and morphological information, employing a genetic identification approach, in addition to size data, to establish definitively that the majority of the partially-plated individuals collected in Lough Furnace were in fact juvenile fish belonging to the completely-plated group, which had yet to develop their full lateral plates. Morphological traits like lateral plates number, body shape and gill raker length are key population trait attributes that differ between two co-occurring ecomorphs in Lough Furnace. While a comparison of different metabolic traits (standard metabolic rate, maximum metabolic rate and aerobic scope in Chapter 4) and the microbiome composition in Chapter 5, were considered to be good candidates that might reveal additional adaptive differences, they were not as informative in this respect as was hoped. Nevertheless, they did yield interesting results in themselves on a broader scale in relation to environmental salinity when fully marine and freshwater outgroups were considered. Completely-plated marine fish collected in the Muingdoran rock pools did

have significantly higher standard metabolic rate than the fish of either plate type found in the brackish Lough Furnace. When salinity was manipulated in mid-experiment to simulate natural tidal conditions, higher salinities produced higher SMR. It might be speculated that a higher SMR is required to meet the physiological demands of surviving in and reproducing in rock pools of high salt concentration (owing to summer evaporation) with maybe different avoidance predator strategy, compared to lower salinity concentrations and different predation pressures in a tidal lagoon. Pursuing this line of investigation and considering the demands that might be associated with courtship behaviour and reproduction, I found that males showing nuptial coloration had significantly higher SMR than males without coloration, suggesting that breeding as indicated by coloration 'costs' a portion of male aerobic scope, possibly as a result of elevated levels of stress.

Based only the morphological traits, low-plated ecomorph observed in Lough Furnace might be fish that migrate from Lough Feeagh into Lough Furnace. However, it was possible using genetic assignment to confirm that a small proportion of the low-plated fish collected in Lough Furnace could be placed within the Rough River and Lough Feeagh genetic groups and hence were in all likelihood individuals originating from the freshwater part of the Burrishoole system either having actively migrated to or been displaced into the lower of the two lakes. However, an assessment of neutral genetic variation indicated that Lough Feeagh low-plated and Lough Furnace low-plated were two discrete genetic populations with likely two independent colonisation histories. As a further insight into the historical relationship between the Lough Feeagh population and the Lough Furnace populations, I found that the freshwater populations had much reduced heterozygosity (genetic variability) as was also reported by Jones *et al*. (2012) and as they suggested, could reflect smaller effective population size and varying

demographic histories involving bottlenecks during the colonisation of freshwater habitats. Interestingly, the brackish populations from the Burrishoole and the marine population from Muingdoran, had similar levels of heterozygosity, and hence, likely, a much larger population base.

It is considered due to the physiography of the catchment between Lough Feeagh and Lough Furnace (embodied by a series of large waterfalls at the Salmon Leap and a high energy mill race) virtually impossible for fish to return back up into upper catchment. Intriguingly, while these Lough Feeagh origin fish could represent a one-way gene flow into either or both of the Lough Furnace stickleback populations, substantial differentiation at neutral genetic markers would suggest otherwise.

Focusing on Lough Furnace, where two ecomorphs were observed, and to attempt to clarify the relationship between them, I undertook an intensive spatial and temporal sampling campaign, and found no obvious spatial (separation by physical distance) or temporal (allochrony) barriers between them. Moreover, both ecomorphs were present in sympatry at the various sampling sites during the breeding season with co-occurring mature females and males of both ecomorphs and with nest building distributed widely throughout Lough Furnace in shallow littoral habitats subject to influence from both high salinity and low salinity. Ravinet *et al*. (2015) had previously sought to resolve using neutral and quantitative trait loci (QTLs) microsatellites whether, what he had considered was a high level diversity in Lough Furnace, had arisen as a result of introgression or reproductive isolation between the divergent completely-plated and low-plated forms; finding strong genetic support for the latter hypothesis. I found little evidence either morphologically (apart from a few outgroups) or genetically to suggest that Ravinet's contention of low level hybridisation was probably incorrect. Moreover, the high level of genetic stability in both low-plated and completely-plated populations over the 12-

years period between 2008 and 2020 – at least four generations assuming generally throughout northern Europe a three-year life cycle (Jones *et al*.2012) – further supports the contention that while the two ecomorphs co-occur at spawning time in Lough Furnace, they are not hybridising. The few individuals, maybe less than 1 or 2%, that could not be definitively assigned to a genetic group and that might reasonably be considered to be hybrids, is very much lower than observed in other similar studies (e.g., Jones *et al*. (2006)), where typically 30% fish collected might be hybrid progeny. Yet despite evidence to the contrary from the characterisation of the populations *in natura*, I undertook a breeding experiment under semi-controlled conditions where females and males from each of Lough Furnace ecomorphs where kept together, viable hybrid offsprings were produced. Low-plated males produced nests and viable offspring with both completely-plated and low-plated females, whereas, conspicuously, completely-plated males produced no offspring. This experiment demonstrated that hybridisation is possible, at least in one direction between the two Lough Furnace ecomorphs, even if due to the limitations of the experimental design, no strong conclusions can be assessed in regard to hybridisation in the wild. The general absence of hybrid individuals suggests that either or both pre- and post-zygotic reproductive barriers must be in operation. Habitat isolation, though on the evidence of the surveys carried out here apparently not a factor, and mate choice have regularly been proposed previously as strong pre-zygotic barriers to gene flow among various ecomorph pairs (Lackey & Boughman, 2017). Alternately, disruptive selection operating on maladapted intermediate ecomorphs, could also act as a possible post-zygotic barrier to gene flow and would be a valuable avenue for further investigation. The successful production of viable offspring maybe lessens the argument for a major role for gametic incompatibilities, at least for one specific pairing.

Following a comprehensive all year round sampling campaign, I found that completely-plated individuals, apart from a small number of fish that were probably juveniles, were absent from Lough Furnace outside of the breeding season, in contrast to the behaviour of low-plated fish which could be found resident in the lagoon all year round. This is convincing evidence for an anadromous or an anadromous-like life history and spawning behaviour (as not fully freshwater) among the completely-plated population. A strict definition of anadromous/anadromy would be the phenomenon whereby a fish is born in freshwater migrates to sea to spend a large part of its life and returns to freshwater to spawn (see Ahnelt, (2008)). There must be a cost versus benefit trade-off in adopting a migratory type behaviour; the presumably dietary advantages of going to sea against risk of predation and subsequently, the advantages of spawning in protected lagoon and lake habitats. I believe this behaviour is the key discriminating trait underpinning the divergence between low-plated and completely-plated stickleback in Lough Furnace. Since the suggestion from this study is that the completely-plated are partially anadromous it begs the question as to whether the selection is occurring in the ocean or in Lough Furnace. The high level of temporal genetic stability found between samples of the completely-plated population collected in this and Ravinet *et al*.'s (2015) earlier study indicates that these fish must be going to sea and home back with considerable fidelity to spawning areas in Lough Furnace. So when thinking ostensibly of completely-plated and low-plated fish occurring in sympatry, they are not really co-occurring. It might be more accurate to suggest that they occur together transiently during the breeding season. If this is the case, it is not unreasonable to speculate that the divergent selection of traits is associated with the time and the selective pressures that these completely-plated stickleback encounter while they are at sea and hence rather than evolution in sympatry in Lough Furnace, heritable changes also result from evolution in allopatry. However, it

might be argued that it is the co-occurrence at breeding time and hence scope for gene flow that really defines sympatry, at least in how reproductive isolation evolves. Concomitantly, the low-plated population is evolving or has evolved in response to the less saline and heavily freshwater influenced conditions in Lough Furnace and might go some way to accounting for the similarities in ecomorph between Lough Furnace and Lough Feeagh. A combination of neutral and non-neutral markers have the potential to elucidate different aspects of the genetic underpinnings of inter-population morphological differences in different environments (Orsini *et al.*, 2013). In this regard, I analysed the two types of markers separately and found that the putatively neutral makers differentiated mainly ecomorphs based on their environment: freshwater vs brackish-marine samples, revealing that brackish samples are more similar to marine samples. In contrast, putatively adaptive markers (mostly driven by two specific markers associated with salinity tolerance and metabolism) differentiated individuals based on their number of lateral plate: low- vs completely-plated regardless of their origin. These results suggested a scenario of isolation-by-environment rather than isolation-by-distance. The putatively functional markers were driven by two SNPs in particular associated with salinity tolerance (chrI_21951727) and metabolism (chrXIV_11360680) genes; though there was also some indication that the previously considered neutral SNP 27027 (chrII_13068160) could also differentiate low-plated and completely-plated in Furnace. Distinctly, 27027 discriminated between the low-plated freshwater samples and the low-plated brackish individuals, but remarkably did not differentiate between the freshwater samples from Lough Feeagh and the Rough river and the completely-plated Lough Furnace fish. It would appear that the 27027 marker is not associated with, or consistently differentiate fish found in Lough Furnace or Lough Feeagh or between

completely-plated and low-plated individuals. It maybe that the Furnace low-plated fish differ from the freshwater fish in some other unknown phenotypic aspect of their biology.

Overall, these findings showed a little amount of parallel evolution as only two of 10 adaptive markers selected from the literature differentiated ecomorph (lateral plate number), whereas neutral markers suggested an isolation-by-adaptation pattern (differentiated by their original environment). This lack of parallelism in marker effect, or at least markers associated or linked to genes that might affect a trait, could be attributed to some extent to ascertainment bias. Ascertainment bias refers to a scenario where a genetic marker panel is most effective in the location where it was developed. Many adaptive variants may exist that are specific to an individual population (Jones *et al.*, 2012) and this seems very much to be the case here. This, quite unexpectedly, given impact on stickleback genetic research, was also the case in the previous study of intraspecific variation carried out by Ravinet *et al*. (2015), where despite targeting five microsatellite loci, putatively linked to QTLs for lateral plate morphology within the Ectodysplasin (EDA) gene, found that their neutral and adaptive microsatellite markers were strongly correlated. In contrast to my study here, for at least two, possibly three (27027 previously presumed neutral) of the selected markers, there was no correlation and hence told a different story. Given, the thousands of neutral and putatively adaptive markers available for stickleback, to find two markers that were not correlated and provided different insights into the divergence of the different Burrishoole population, though targeted from the literature, was still somewhat fortunate.

By pulling all the results together and speculating to some extent, two different populations that were morphologically differentiated were found to be present together in sympatry in Lough Furnace during the breeding season, and yet were genetically different at both neutral and adaptive markers and apparently were so much substantially

adaptively diverged as to not produce hybrids, despite being sexually mature and captured together. Perhaps these populations have evolved in two different environments (allopatry) – one freshwater/brackish; one fully marine – with the anadromous completely-plated fish collected in Lough Furnace evolving in a purely marine environment, and the low-plated population in a brackish environment or possibly the freshwater parts of the lake.

Candidate 'abiotic factors' that might be important in a isolation-by-environment scenario in Lough Furnace would include: current and past salinity levels; salinity gradient across transitional waters; salinity gradient variability in respect of short and long cycles (e.g. tidal and seasonal) and freshwater inputs and temperature regimes across transitional waters; climate variability (including climate change) in respect of freshwater hydrological and temperature regimes; and finally, over millennials, sea level variation and contemporaneous and historical catchment physiography in respect of glaciation cycles.

Future Research

While studying the genetic structure of ecomorphs in the Burrishoole (Chapter 3), I had access to only 30 SNPs to assess the population genetic structure. This number of markers would be considered to be rather low, especially as SNPs are bi-allelic markers and lack the variation that would be associated with compared to microsatellite markers. The adaptive markers selected from the literature (Jones *et al.*, 2012a; Ferchaud *et al.*, 2014b) showed patterns of divergent selection, and thus, differentiated freshwater and marine populations in different geographic systems. However, it was not unexpected (because of ascertainment bias) that these markers might not be under divergent selection in this specific system (especially the eight adaptive showing patterns similar to the neutral

markers). In respect of ascertainment, freshwater and marine populations selected by Jones *et al*. (2012) were distributed around the Pacific Ocean (Japan, Alaska, British Columbia) and Atlantic Ocean (Iceland, Scotland, Norway and Germany). In Ferchaud *et al.* (2014), freshwater and marine stickleback populations were sampled in Denmark. These eight markers were not effective discriminators in the Burrishoole. Moreover, I did not have any markers associated to the number of lateral plate in this study (*EDA*), as the development of the assay for these markers did not work. For future work in this system, it would be useful to apply different genomic methods to find which, potentially geographically-specific, regions of the genome are the most differentiated between these ecomorphs, such as RAD-sequencing or whole genome sequencing (WGS).

I was able to exclude certain barriers in my morphological study described in Chapter 2 such as coarser-level spatial and temporal separation, as both completely-plated and low-plated ecomorphs were present in Lough Furnace at the same time, and often in the same traps. While I tested the possibility of producing hybrids in a semi-controlled environment, leaving the fish to breed freely into a tank, this experiment was not designed to test mate choice *per se*. A follow-up to this would be a mate-choice experiment in which females were able to observe physically-partitioned males from two or more ecomorphs at the same time (Milinski *et al.*, 2005; Andreou *et al.*, 2017; Gahr *et al.*, 2018). This could be extended to test for passive or active chemical signalling cues via a design in which females could not see males but could 'smell' them via water flow. For future work, it would also be interesting to assess the effect of alternative reproductive barriers that could occur in this system, as it is common that many reproductive barriers contribute to the maintenance of reproductive isolation. In lake-stream pairs of sticklebacks, Hanson *et al*. (2016) showed that allochrony (spawning at different times) contributed from 8-39% of reproductive isolation between pairs. In another example from

the plant species monkeyflowers (*Mimulus* spp), nine different barriers have been identified as contributing to reproductive isolation (sometimes with contrasting effect), and the reproductive isolation was almost total (Ramsey & Bradshaw, 2003).

The main limit for the metabolic work described in Chapter 4 was not being able to assay all ecomorphs for all metrics. I also did not collect metabolic traits from the Lough Feeagh ecomorph; as Feeagh fish of suitable size were relatively scarce compared to the size of the chambers available. Due to constraints on availability of the respirometry equipment and uncertainty around resolving these acclimation issues, I chose to focus collection and respirometry effort on the other ecomorphs. Therefore, future research on metabolic traits in this system should include sticklebacks from Lough Feeagh, as they would provide an important baseline for the purely freshwater ecomorph. Also, future work should include more respirometry from outside of the breeding season so as to measure metabolic traits when individuals were not in a reproductive state, though this is caveated by the difficulty of catching the marine and completely-plated ecomorphs outside of the breeding season. Moreover, it would be informative to capture juveniles from different ecomorphs and measure their metabolic rates though this would require respirometry equipment capable of measuring smaller fish than was available in the setup. A measure of metabolic capacity while fish are growing could also bring information in respect of the potential cost of growing plates between low- and completely-plated.

In the analyses of gut microbiome (Chapter 5), the sample sizes of some groups were relatively small, especially for environmental samples. For the marine environmental samples, there were only two samples available. For future work, sampling juveniles, especially for completely-plated in Lough Furnace, would give potentially some important insights into the impact of migration to sea during winter and their return for

the breeding season, as it would show if the microbial communities colonising the gut in Lough Furnace are persistent during their migration in marine environment. Similarly, investigating the gut microbiome of low-plated resident in Lough Furnace, would be necessary to measure the potential seasonal shifts from winter to spring/summer of the communities present in Lough Furnace. However, one of the most challenging aspects in microbiome studies is to establish which communities are transient and which persist in the host gut. A powerful extension in future work would be to separate the different parts of the gut (content vs tissue, stomach vs mid-gut), as well as prey microbiome. To understand how the host genotype filters the communities, it might be relevant to run transplant experiments in gnotobiotic individuals (bacteria-free lineage). An example could be in gnotobiotic sticklebacks, as it has previously be done in marine and freshwater sticklebacks marine and freshwater reared in a common intermediate salinity environment (Milligan-Myhre *et al.*, 2016).

A further in depth study of the migratory behaviour of the completely-plated fish form Lough Furnace would be very valuable. Therefore, a number of investigating strategies would be useful such as the application of the various modern tagging methods, for example PIT tagging with antennas could be effectively deployed in the system. Laos, to reconstruct their migration pathways, using stable isotopes retrieved from the muscle, lateral plates or otoliths could reveal some insights.

Summary

Overall, my thesis sought to improve understanding of intraspecific variation in different populations characterised by ecomorph diversity when found in parapatry and sympatry, and to consider how this diversity is maintained. To bring some insights to this complex problem, I undertook a multivariate approach to precisely characterise intraspecific

variation of the different ecomorphs in the Burrishoole ecosystem complex and beyond (the marine rock pools at Muingdoran and the few extra specimens collected on the Hebrides). The results described in this thesis offer some solid phenotypic and genetic background of different local ecomorph specificity, in respect to the selective pressures in their environment and might elucidate the processes involved in ecological speciation. Through establishing what potential factors drive and maintain the intraspecific diversity, my work also brings insights into the mechanisms which can lead ultimately to ecological speciation such as reproductive isolation, adaptive divergence and genetic drift. More work is required to fully understand all the mechanisms that restrict or prevent hybridisation in this system. The research outlined in my thesis goes some way to reveal the phenotypic and genetic complexity inherent in different stickleback ecomorphs, and how their biology and life-history and the various environments within which they live have shaped this diversity.

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Amendments post-viva

This is something that was discussed during the design of the experiment and the preparation of the chapter for submission for peer review, but I recognise that I did not adequately convey this in the original version of the thesis, and that I should have presented additional formal testing.

The study was primarily targeted at characterising metabolic traits under approximations of immediately-prevailing environmental conditions in fish fresh from the wild. Fish were captured, acclimatised to captivity for 24 hrs, and measured under the temperature and salinity at capture. Our expectation was that this would provide adequate, natural variation in temperature and salinity to test these as predictors of metabolic rates within each ecomorph. This expectation was borne out for Furnace-caught fish but not for the marine fish, limiting the comparability of the latter to the former.

While there would have been definite benefits to my study of performing trials with fish acclimatised to temperature and salinity conditions set a priori, these would have been additions and not alternatives. I was keen to test fish as near to their prevailing environmental conditioning as possible, and under prevailing variation in those conditions. Fish acclimatised to pre-set conditions would, by definition, be unsuitable, and the tested conditions might even be outside their natural experience. Moreover, we did not have the infrastructure in place for that degree of acclimatisation.

With hindsight, I ought to have performed temperature change trials analogous to those performed for salinity, which would have both created more testable variation in the predictor and allowed a degree of attribution of causality.

To address the point raised by the examiners, I sought to fit a model with all three key predictors– ecomorph, temperature and salinity – present simultaneously, and to explore pairwise interactions. Exploratory analyses suggested multicollinearity – assessed via variance inflation factor (VIF) – was problematically high. To help control VIF, all subsequent analyses were performed after z-transforming temperature and salinity to have mean = 0 and SD = 1, and, where necessary, tests for interactions between ecomorph and continuous predictors were repeated with the continuous predictor re-centred within ecomorph. As with previous analyses, all models were linear mixed models with date and bath number as a random effect, and with FUR_CP as the reference ecomorph.

When all three focal predictors were present in the same model without interactions, only temperature was a significant predictor (lmer : $F1 = 4.83$, $P = 0.028$) of SMR. Its effect was positive (slope = 17.3, SE = 7.88, t = 2.2), in agreement with the original analyses. While the non-significance of salinity and ecomorph seems to contradict the original analyses, both predictors showed problematically high VIFs (6.36 and 5.54 respectively) when included together.

Adding an interaction between salinity and ecomorph to the above model was not viable, as it resulted in VIFs of 61.79, 133.03 and 305.41 for salinity, ecomorph, and the interaction respectively. Even when salinity was re-centred within each ecomorph, salinity and the interaction still had VIFs ≥ 11.24. From this, I concluded that my dataset does not allow me to reliably separate potential effects of ecomorph from potential effects of salinity when both predictors are present together, and when the full dataset (i.e., all ecomorphs) is used.

In a main-effects model using temperature and salinity, but not ecomorph, temperature and salinity both had significantly positive relationships with SMR. In a main-effects

model using temperature and ecomorph, but not salinity, temperature had a significant positive relationship with SMR, and ecomorph was a significant predictor of SMR, with marine fish having significantly higher SMR than the reference level. Both of these two models had slightly lower AICC values than the model that included both salinity and ecomorph. These models argue that either marine fish have higher SMR or higher salinity is associated with higher SMR, but that the two predictors are too cofounded within the full dataset to allow any separate effects to be unpicked.

There was no problematic multicollinearity between temperature and ecomorph or between temperature and salinity, and no significant interactions between them. Temperature retained its significant, positive relationship with SMR in all of these models. These results are consistent with the original submission.

Models that included all three key predictors after removal of the marine ecomorph had acceptable VIFs, and did not result in different interpretations from the original submission.

Collectively, while these additional analyses provide a stronger statistical basis for my already-cautious interpretation of the confounding of the marine ecomorph with high-salinity treatments, they do not produce interpretations that diverge from the original submission.