SPECIATION IN THREE-SPINED STICKLEBACK

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

April 2018

ABSTRACT

Speciation, the division of one species into two, has provided evolutionary biologists with a rich ensemble of questions, conundrums and revelations for over a century, and yet our understanding of many of the factors affecting this complex, multidimensional process remains limited. In this thesis, I aimed to further our understanding of speciation using divergent populations of three-spined stickleback (*Gasterosteus aculeatus*) on the island of North Uist, Scottish Western Isles. Firstly, I explored the degree of morphological and genetic separation between three stickleback ecotypes, showing that both strongly reproductively isolated, and admixed populations exist in close proximity. I then attempted to identify the ecological and genetic origins of strongly isolated species-pairs, testing two competing explanations for their existence. I showed that a recent 'double-invasion' is unlikely, but found stark differences in the long-term genetic history between ecotypes, indicating that the evolution of species-pairs may be related to secondary contact between anciently diverged mitochondrial lineages. I then conducted mate choice trials to assess mating preferences between ecotypes, and to test for reinforcement in species-pairs. Consistent with the idea that speciation in this case is not driven purely by ecological factors, I found no evidence that reinforcement drives assortative mating in species-pairs. Rather, it appears that extant mating preferences have developed as a by-product of other adaptations. Finally, I took a brief interlude to document and investigate an exciting chance finding, internally developed embryos retained within the ovaries of a normally oviparous species, before concluding by summing up my findings, their relevance for scientific progress, and avenues opened up for further research.

ACKNOWLEDGEMENTS

First, and foremost, I would like to thank my supervisor Andrew MacColl for his endless patience, enthusiasm and support over the last four years, and for his invaluable advice towards all aspects of this project. Thanks also to my second supervisor Suzanne McGowan and to all members of the MacColl lab: Shaun Robertson, Isabel Santos-Magalhaes, Jim Whiting, Talib Chitheer, Muayad Mahmud, Abdul Rahman, Becca Young, Sarah Evans, Dan D'Agostino, and Mahmuda Begum for help in the lab, on fieldwork and for making this experience a thoroughly enjoyable one, full of fun, laughter and happy memories that I will treasure forever!

Special thanks to all of my office mates, past and present, in B109 for their endless support, advice, friendship and for calming my many stats related tantrums! Thanks to Sarah Goertz, Hannah Jackson, Marie Athorn, Hazel Clarke, and Libertad Sanchez for endearing girly chats (and thanks Jonathan Fenn, Stu Young, Jim Whiting and Dan D'Agostino for suffering them). Thanks to Jim for sharing his excellent stats knowledge and strange taste in music. Particularly, thanks to Dan for scintillating post 4pm conversations that helped to keep me sane and smiling!

Above all, thanks to my dad for his infinite emotional and financial support, without which this thesis would not have existed, and to both my parents for encouraging and inspiring my endless curiosity into the natural world and for teaching me that the sky is the limit and to never give up.

This thesis is for my daughters, Livvy and Caitlyn.

CONTRIBUTIONS

The majority of the work described in this thesis is my own, however a number of people made important contributions to individual chapters as follows:

Chapter 2: Salinity measurements and trapping to determine the occurrence of anadromous stickleback, prior to 2015 was conducted by Andrew MacColl, Daniele D'Agostino, Sonia Chapman, Isabel Santos-Magalhaes and Shaun Robertson. James Whiting aided in the interpretation of the linear discriminant analysis.

Chapter 3: Sediment core samples from 2013 were collected by Xu Chen and Suzanne McGowan. Suzanne McGowan aided in the interpretation of sediment analyses. Gareth Lee assisted with collecting loch elevation data, analysed the data and produced elevation maps. All freshwater and a few brackish stickleback mtDNA sequences were obtained by Rahn *et al.* (2016). Macrofossil samples were prepared to graphite by the East Kilbride NERC Radiocarbon Facility NRCF010001 (allocation number 1942.1015) and dated by the SUERC AMS Laboratory.

Chapter 4: Andrew MacColl helped to design the experiment, Andrew MacColl, James Whiting, Amelia Reddish and Mahmuda Begum assisted in the collection and husbandry of stickleback in breeding condition. Amelia Reddish conducted some of the mate choice trials in 2016.

Chapter 5: Samples of gravid females were collected by Andrew MacColl and Job de Roij. Andrew MacColl carried out dissection of the internally fertilised stickleback. Shaun Robertson conducted the majority of sex testing PCRs and

all other microsatellite analysis and aided in interpretation of the results. Muayad Mahmud sectioned and mounted gonadal tissues and Muayad Mahmud and Abdul Rahman assessed slides for the presence of testicular tissue in the internally fertilised female.

Andrew MacColl aided in the analysis and interpretation of results, and commented on conceptual ideas for all chapters.

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CHAPTER 1 : GENERAL INTRODUCTION

1.1 What is a species?

There are numerous different definitions of what constitutes 'a species' (Zinner and Roos, 2014), and arguments regarding where to draw species-boundaries have been ongoing since the formation of evolutionary theory (Bird *et al.*, 2012; Britton, 1908; Coyne *et al.*, 1988; Darwin, 1859; Hart, 2011; Lamarck, 1809). This has been particularly problematic for researchers interested in speciation because difficulties defining what species are make it almost impossible to define the processes responsible for their formation (Bird *et al.*, 2012; Hausdorf, 2011). Historically, species were classified based on morphological similarity; the 'Typological Species Concept' (Mayr, 1942), but with the increasing observation of both cryptic species and those with strikingly variable intraspecific characteristics e.g. strong sexual dimorphism, multiple reproductive strategies, or seasonal or age related phenotypic differences (Brakefield and Reitsma, 1991; Coleman *et al.*, 1994; Oug, 1990; Parker, 1992), it became clear that this definition was inadequate. With a growing understanding of genetics and the mechanisms of inheritance, the importance of reproductive isolation (RI) in distinguishing species was realised, and the 'Biological Species Concept' (BSC) became established (Dobzhansky, 1937; Mayr, 1942). This defines biological species as *"groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups"* – Ernst Mayr (Mayr, 1942; 2000), and, although a plethora of other species concepts have been proposed over the last few decades (Hausdorf, 2011; Howard and Berlocher, 1998), the BSC remains the most

popular among researchers interested in sexually reproducing, natural populations (Cicconardi *et al.*, 2013; Koffi *et al.*, 2010; Liu *et al.*, 2012; Sokal and Crovello, 1970).

The emphasis of RI in distinguishing species boundaries is useful, but it makes hybridisation and introgression between species, and partial isolation between distinct ecological morphs, varieties and geographical races within species, problematic (Mallet, 2005). How many hybrids must be 'accidentally' produced between divergent populations before they should be classified as one species rather than two? And how much RI is necessary to define individual species? Low levels of hybridisation between recognised species are relatively common in nature (Grant and Grant, 1992; Noor *et al.*, 2000), particularly in plants (Griffin *et al.*, 1988; Hardig *et al.*, 2000), and divergent ecotypes can be almost completely reproductively isolated and yet still be classified as a single species (McKinnon and Rundle, 2002; Nosil, 2007; Taylor and Bentzen, 1993). If hybridisation can take place above the level of species, and partially isolated morphs or races can be evident below the level of species, it is arguable that species do not really exist as distinct, categorical entities in the way that biologists have traditionally imagined (Dobzhansky, 1937; Mayr, 2000). Rather, they represent a stage, not necessarily an endpoint, within a continuous process (Hendry, 2009; Nosil *et al.*, 2009).

The idea that speciation is more appropriately described as a continuum, than a series of jumps between distinct states has become increasingly popular as it allows for a much more accurate evaluation of the state of isolation between populations (Hendry *et al.*, 2009; Mallet, 2008; Powell *et al.*, 2013). Speciation

as a continuum shifts the focus away from trying to rigidly define species and raises new questions about where populations fall along this gradient of divergence, how they got there, and why their isolation has not progressed further (Hendry *et al.*, 2009). These are the questions on which this thesis is based.

1.2 Causes and contexts of speciation

1.2.1 Geographical contexts

Speciation has most commonly been classified into modes based on the geographical context under which divergence has occurred (Slatkin, 1987; White, 1978). Broadly, this encompasses: allopatry, describing populations between which contact is completely prevented by geographical barriers; parapatry, referring to partially geographically isolated populations with limited contact owing to partial range overlap; and sympatry, whereby populations are in direct contact with one another throughout their ranges (Butlin *et al.*, 2008; Fitzpatrick *et al.*, 2009). These classifications describe spatial scenarios, but their importance for speciation is largely related to the amount of possible gene flow between populations, with sympatry describing complete gene flow, allopatry, no gene flow, and parapatry, somewhere in-between (Bolnick and Fitzpatrick, 2007; Mayr, 1963; Nosil, 2008). Such classifications have fuelled a wealth of valuable research and debate over the years, particularly with regards to understanding the most likely conditions under which speciation takes place (Barraclough and Vogler, 2000; Losos and Glor, 2003), and how it might be possible despite the homogenising effects of gene flow under sympatric and parapatric conditions (Barluenga *et al.*, 2006; Dieckmann and Doebeli, 1999;

Doebeli and Dieckmann, 2000; Savolainen and Vepsalainen, 2003; Via, 2001). However, such discrete categorisation of what is a long and potentially extremely complex process has been heavily criticised for its oversimplicity (Butlin *et al.*, 2008; Fitzpatrick *et al.*, 2008; 2009; Gavrilets, 2003; Gould, 1980; Via, 2001). These classifications are inadequate in that they divide a 'gene flow continuum' into discrete states, causing researchers to focus disproportionally on the extremes (Via, 2001), and they do little to allow for other dimensions of variability, or the possibility that populations may experience a variety of geographical contexts during speciation (Grant and Grant, 2009). Nonetheless, geographical context, and thus opportunity for gene flow, is important for understanding divergence. But shifting the focus away from trying to rigidly define geographical categories, and concentrating on the evolutionary mechanisms responsible for speciation, and how factors such as gene flow and selection interact with spatial distributions, will ultimately lead to a better understanding of the causes and contexts of speciation (Fitzpatrick *et al.*, 2009).

1.2.2 Evolutionary mechanisms

If populations are evolving as separate entities, without exchanging genes, RI can easily develop via random genetic processes or events, such as genetic drift or whole genome duplication (Devaux and Lande, 2008; Mallet, 2007; Orr, 1991; Wood *et al.*, 2009). Furthermore, adaptive divergence, under uniform selection, can also lead to speciation if different advantageous (but incompatible) mutations happen to evolve in separate, genetically isolated populations (Turelli *et al.*, 2001). Bateson (1909) proposed the simplest model of the genetic basis for random modes of speciation, which was later extended by Dobzhansky

(1937) and Muller (1942), to become known as 'Bateson-Dobzhansky-Muller (BDM) incompatibilities'. This model involves two loci that evolve from an ancestral genotype of $A^1A^1B^1B^1$ to $A^2A^2B^1B^1$ in one population and $A^1A^1B^2B^2$ in another. Speciation results, or is at the very least initiated, if, on secondary contact, epistatic interactions between alleles A^2 and B^2, which have never needed to be compatible, lead to hybrid inviability, sterility or reduced hybrid fitness (Doi *et al.*, 2001; Hu *et al.*, 2016; Sturtevant, 1956). In reality, the genetic architecture of speciation is rarely this simplistic (Ting *et al.*, 2001; Wu and Palopoli, 1994; Zeng *et al.*, 2000), but the theory stands regardless of the number of 'speciation genes' involved. The potential for speciation to occur as a result of random processes, in the absence of gene flow, is largely uncontentious (Adamowicz *et al.*, 2009; Near and Benard, 2004). However, there remains considerable debate regarding the proportion of speciation in nature that is accounted for by this mechanism (Barraclough and Vogler, 2000; Nosil, 2008; Turelli *et al.*, 2001).

If there is gene flow between populations, speciation by random mechanisms becomes almost impossible, and, as a result, speciation with gene flow, or 'sympatric speciation', is a highly contentious topic (Berlocher and Feder, 2002; Bird *et al.*, 2012; Bolnick and Fitzpatrick, 2007; Coyne and Price, 2000; Dieckmann and Doebeli, 1999; Doebeli, 1996; Doebeli and Dieckmann, 2000; Fitzpatrick *et al.*, 2008; Higashi *et al.*, 1999; Via, 2001). Gene flow has a homogenising effect (Coyne and Orr, 2004) and the random fixation of singularly beneficial, but mutually detrimental alleles is prevented by natural selection (Mayr, 1963). Such alleles could only prevail with gene flow if they were associated with traits that were differentially adaptive in different

environments, and thus under divergent or disruptive natural selection (Bird *et al.*, 2012). This process, termed 'ecological speciation', is, therefore, theoretically possible regardless of the level of gene flow between divergent populations (Schluter, 2001), and is generally associated with much more rapid divergence than speciation by random mechanisms, because of the role of natural selection (Hairston *et al.*, 2005; Hendry *et al.*, 2007; Orr and Orr, 1996). Ecological speciation requires a source of ecologically-based divergent selection, which includes selection on both biotic interactions with other species e.g. predation or resource competition, and abiotic components of both habitats e.g. resource availability, climate and physical and chemical structure. It also requires a mechanism of reproductive isolation e.g. pre-zygotic barriers such as temporal, behavioural, spatial or mechanical isolation, and/or post-zygotic barriers such as hybrid inviability, sterility or reduced hybrid fitness, and a genetic means of linking divergence in traits under selection with reproductive isolation (Rundle and Nosil, 2005).

Ecological speciation has become extremely popular in recent years (McKinnon *et al.*, 2004; Nosil, 2012; Orr and Smith, 1998; Rundle and Nosil, 2005; Schluter, 2009; Wiens, 2004), and there is convincing evidence for its occurrence, both from manipulative experiments (Rice and Hostert, 1993) and in nature (Filchak *et al.*, 2000; Jiggins *et al.*, 2001; Martin and Willis, 2007). Three-spined stickleback (*Gasterosteus aculeatus*), offer particularly good examples, having repeatedly evolved reproductively isolated ecotypes, associated with divergent selection based on ecological differences (Hatfield and Schluter, 1999; McKinnon *et al.*, 2004; Nagel and Schluter, 1998; Rundle *et al.*, 2000; Schluter, 1996; Schluter and McPhail, 1992). However, many examples of ecological

speciation, including stickleback, describe partial, rather than complete RI (Hendry, 2009), suggesting that whilst ecological factors clearly drive divergence, and may often initiate speciation even with gene flow, the potential for speciation to progress to completion under these conditions may be limited.

1.2.3 What can mitochondrial DNA (mtDNA) tell us about speciation?

In order to infer causes and contexts of speciation, it is often necessary to estimate the evolutionary history, and relationships among populations or species. This can be done through phylogenetic inference (Swofford *et al.*, 1996). The field of phylogenetics has grown exponentially over the last few decades, with the increasing ease with which large amounts of genetic data can be obtained (Castresana, 2000; Chen and Du, 2018; Clermont *et al.*, 2000; Dunn *et al.*, 2008; Iwabe *et al.*, 1989; Takezaki and Nei, 1996; Tatusov *et al.*, 2001). Mitochondrial DNA (mtDNA) possesses a number of (supposed) characteristics which make it an ideal marker for species delimitation and phylogenetic analyses (Avise *et al.*, 1987), and it has been widely utilised for decades for this purpose (Foote *et al.*, 2009; Hasegawa *et al.*, 1985; Irwin *et al.*, 1991; Makinen and Merila, 2008; Miya *et al.*, 2003; Morin *et al.*, 2010; Simon *et al.*, 1994).

In almost all animals, mtDNA is exclusively maternally inherited (Birky, 2001), meaning it should bypasses any opportunity for recombination. This is particularly useful for phylogenetic reconstruction, for which recombination can be a major issue (Avise *et al.*, 1987; Poke *et al.*, 2006; Posada and Crandall, 2002; Schierup and Hein, 2000). However, mitochondria possess all of the biochemical apparatus necessary for recombination (Thyagarajan *et al.*, 1996), and it is becoming clear that recombination has occurred in some species

(Gantenbein *et al.*, 2005; Hoarau *et al.*, 2002; Ladoukakis and Zouros, 2001; Ujvari *et al.*, 2007), and even occasionally between species (Ciborowski *et al.*, 2007), casting doubt on the suitability of mitochondrial genes as single phylogenetic markers (Ballard and Whitlock, 2004; White *et al.*, 2008). MtDNA has also long been assumed to be exempt from the actions of natural selection, and thus was expected to evolve neutrally, with a clock-like rate of nucleotide substitution that should approximately reflect divergence times (Gissi *et al.*, 2000; Martin, 1995). However, mitochondrial functions are vital for survival as, amongst other things, mtDNA encodes a group of proteins which must interact with proteins derived from nuclear DNA to form the necessary apparatus for oxidative phosphorylation and cellular respiration (Blier *et al.*, 2001; Taanman, 1999). Mutations that affect the expression of mitochondrial proteins are associated with severe pathologies known as 'mitochondrial disease' (Lin and Beal, 2006; Pearce *et al.*, 2017; Wallace, 1999), illustrating the direct effects of mitochondrial function on fitness. A number of studies have also identified differential fitness across mitochondrial haplotypes (Ballard *et al.*, 2007; De Stordeur, 1997; Foote *et al.*, 2011), along with genetic signatures of positive selection (Bazin *et al.*, 2006; Castoe *et al.*, 2009; Rodell *et al.*, 2013), and highly variable rates of nucleotide substitution (Allio *et al.*, 2017; Nabholz *et al.*, 2008; 2009; Xu *et al.*, 2006). This suggests that the evolution of mitochondrial genes is far from neutral, and that phylogenetic inference based purely on these markers may be inherently flawed (Ballard and Whitlock, 2004; Galtier *et al.*, 2009; Havird and Sloan, 2016; White *et al.*, 2008).

Whilst the non-neutrality and non-uniform substitution rate of mtDNA questions the heavy reliance on this marker alone in phylogenetic analyses, it raises new

questions regarding the ecology and evolution of mitochondria. Given the importance of the role played by mitochondria for energy production and metabolism, it is highly likely that mitochondrially-mediated changes are important in ecological adaptations to different environments. Furthermore, the differences in modes of inheritance between nuclear and mitochondrial genomes could cause significant, conflicting differences in fitness between sexes, driven by sexually antagonistic selection (Connallon *et al.*, 2018; Hill, 2018; Matessi and Saino, 2003). Although the importance of the mitochondria, and the role of mitonuclear interactions and coadaptations in speciation is only just beginning to be realised (Hill, 2016; 2017; 2018; Sloan *et al.*, 2017), it provides an exciting new avenue for evolutionary research.

1.3 Fluctuations in spatial distributions during speciation

The fact that speciation is a process, rather than an event means that, in reality, many examples of speciation involve multiple stages of spatial isolation, encompassing differing levels of gene flow between populations (Barraclough and Vogler, 2000; Martin-Bravo *et al.*, 2010). Range shifts often result in secondary contact between previously allopatric populations that have evolved partial RI already, via any evolutionary mechanism (Quenouille *et al.*, 2011). The outcome of hybridisation on secondary contact can be: extinction of one of the two populations, homogenisation of two populations into one, or, if hybrid fitness is significantly lower than that of either parent population, it can also lead to exaggerated RI and the advancement of speciation by reinforcement (Blair, 1974; Liou and Price, 1994). Reinforcement is the enhancement of assortative mating, i.e. a preference for mating with conspecific over heterospecific

individuals, as a result of natural selection to reduce maladaptive hybridisation (Blair, 1955; Dobzhansky, 1937). It is most likely when assortative mating is based on traits which are also under divergent selection, sometimes referred to as 'magic traits' (Butlin and Smadja, 2018; Gavrilets, 2004; Servedio *et al.*, 2011), or when the genes underlying both assortative mating and divergent selection are in linkage disequilibrium (Servedio, 2000; 2009). Reinforcement often results in amplification of traits under divergent selection, known as 'character displacement' (Brown and Wilson, 1956; Slatkin, 1980), as well as increased assortative mating, and these factors are commonly used as evidence for its occurrence (Gerhardt, 1994; Saetre *et al.*, 1997). The high incidence of secondary contact in nature (Campagna *et al.*, 2014; Moritz *et al.*, 2009; Ravinet *et al.*, 2013; Sa-Pinto *et al.*, 2010), indicates that reinforcement may be common, however, the frequency with which it occurs remains debated (Abbott *et al.*, 2013; Garner *et al.*, 2018; Servedio, 2004). Identifying reinforcement can also be difficult because it can occur without leaving the signature of reproductive character displacement (Garner *et al.*, 2018; Lemmon *et al.*, 2004), and assortative mating can develop as a by-product of other adaptations, without the influence of reinforcement (Rice and Hostert, 1993; Vines and Schluter, 2006).

Although it is accepted that secondary contact (and therefore changes in spatial distributions and opportunities for gene flow) is common during speciation, it is often ignored or underplayed in speciation research (Chesser and Zink, 1994; Doebeli and Dieckmann, 2003; Foote, 2018; Grant and Grant, 2009; Moura *et al.*, 2015). This may well be because the two major components involved in investigating such changes: an estimation of the timeframe during which divergence took place, and a reconstruction of the spatial distributions of

populations throughout the same timeframe, are both notoriously difficult to acquire (Fitzpatrick and Turelli, 2006; Ho and Phillips, 2009; Sanderson and Shaffer, 2002). Estimates of the timings of cladogenesis, inferred from species-level phylogenies (Barraclough and Vogler, 2000), are plagued by uncertainties regarding the most accurate methods for phylogenetic reconstruction (Gadagkar and Kumar, 2005; Kolaczkowski and Thornton, 2004), molecular clock calibrations (Bell, 2015; Dornburg *et al.*, 2014; Duchene *et al.*, 2014; Graur and Martin, 2004; Ho and Duchene, 2014; Ho *et al.*, 2008; Schenk and Hufford, 2010), and, particularly for recently diverged populations, discordance between gene and species trees or 'incomplete lineage sorting' (Degnan and Rosenberg, 2009; Ho and Phillips, 2009; Maddison and Knowles, 2006). Reconstructions of historic spatial distributions are also problematic. Many models assume that current distributions can be used to infer past geographical population boundaries, largely ignoring the possibility of temporal range shifts. (Barraclough and Vogler, 2000; Losos and Glor, 2003). However, there is a wealth of data which demonstrates that this assumption is almost never met, as populations readily experience range changes as a result of climatic and environmental fluctuations (Corser *et al.*, 2012; Mimura *et al.*, 2014; Quenouille *et al.*, 2011; Watts *et al.*, 2013). The application of an interdisciplinary approach can, in some situations, alleviate this problem and allow past changes in habitat connectivity to be used to infer historic population distributions (Graham *et al.*, 2004).

1.4 Relative sea-level (RSL) change and speciation

Fluctuations in relative sea-level (RSL) occur almost constantly, and can dramatically alter the structure and connectivity of coastal and shallow marine habitats. This has a huge impact on the inhabitants of such environments, and frequently causes population bottlenecks (Fauvelot *et al.*, 2003; Ludt and Rocha, 2015), extinctions (Hallam, 1989; Hallam and Wignall, 1999; Woodroffe and Grindrod, 1991) and range shifts, that have contributed to speciation (Beheregaray *et al.*, 2002; Lessios *et al.*, 2001; Palumbi, 1994; Rundle and Schluter, 2004; Shen *et al.*, 2011; Taylor and McPhail, 2000). For radiations involving a marine to freshwater transition, RSL change reconstructions can be used to infer past connectivity between coastal habitats, and thus periods where gene flow was possible, and when it was prevented.

1.4.1 Methods for RSL change reconstruction

Instrumental records of changes in RSL can only provide data spanning just over a century (Church and White, 2011), and thus palaeo data are required in order to understand RSL change over the timescales generally necessary for speciation to take place (Gavrilets, 2000; Good-Avila *et al.*, 2006; Sepkoski, 1998; Stephens and Wiens, 2003). Detailed records of past climatic changes and environmental conditions are stored in sediment deposits at the bottom of lakes and coastal lagoons (Hodell *et al.*, 2005; Ladwig *et al.*, 2017; Meng and Liu, 2018; Peng *et al.*, 2005; Schmidt *et al.*, 1990; Wright, 1980), and various proxies for salinity can be used to reconstruct historic marine influxes and transitions (Berben *et al.*, 2017; Espinosa *et al.*, 2012; Holmes *et al.*, 2007; Kotthoff *et al.*, 2017; Li *et al.*, 2015; Mertens *et al.*, 2009). Several macrofossil assemblages are

preserved well enough in palaeo-sediment for this purpose, particularly foraminifera and diatoms (Espinosa *et al.*, 2012; Saunders, 2011; van Soelen *et al.*, 2010). Diatoms are a particularly good proxy for salinity as many taxa have distinct and limited tolerances to salt content (Horton *et al.*, 2006; Jiang *et al.*, 2014; Juggins, 1992; Saunders, 2011; Taukulis and John, 2009; Van Dam *et al.*, 1994; Vos and Dewolf, 1993), however there are a number of issues associated with utilising ancient diatom assemblages for this purpose. The calibration of predicted salinity tolerances of ancient diatom species involves using transfer functions generated from modern species-environment relationships (Zong and Horton, 1999). The assumption that the tolerances of modern day analogues mirror those of ancient diatom populations is somewhat problematic, as it ignores the possibility of adaptations to different salinities over time (Alverson, 2014; Alverson *et al.*, 2011; Ketola and Hiltunen, 2014), as well as the effects of other environmental variables, such as temperature (Chen and Stillman, 2012; Jacobsen *et al.*, 2007), nutrient gradients (Lee *et al.*, 2013) and pH (Berezina, 2001) on salinity tolerance. Furthermore, in some cases modern analogues no longer exist at all (Kemp *et al.*, 2009; Wilson and Lamb, 2012).

Inferences about historic changes in climate and environmental conditions can also be made by studying the elemental composition of lake sediment directly using energy dispersive X-ray fluorescence, or 'ED-XRF' (Makundi, 2001; Szaloki *et al.*, 1999; Tung, 2004), although to date the application of this method has largely been restricted to identifying heavy metal pollution (Araujo *et al.*, 1998; Ravisankar *et al.*, 2015; Tholkappian *et al.*, 2018). This method has the advantage of bypassing many of the biological issues associated with macrofossil proxies as transfer functions and modern analogues are not

necessary. In reality, reconstructions that utilise a multi-proxy approach are likely to provide the most reliable and accurate estimations of historical salinity fluctuations (Berben *et al.*, 2017; Hodgson *et al.*, 2005; Holmes *et al.*, 2007; Kotthoff *et al.*, 2017).

1.5 Speciation in stickleback

Three-spined stickleback (*Gasterosteus aculeatus*, hereafter 'stickleback'), are small, oviparous (egg-laying), teleost fish (Figure 1.1) which are common in both marine and freshwater habitats throughout the Holarctic (DeFaveri *et al.*, 2011). Stickleback are a model organism for studying speciation (McKinnon and Rundle, 2002; Schluter, 2001). Following the retreat of the Pleistocene glaciers circa 10,000 to 20,000 YBP (Ballantyne, 2010), ancestral marine/anadromous stickleback have colonised many newly formed freshwater habitats repeatedly and independently across their range (Jones *et al.*, 2012b). During this, evolutionarily speaking, extremely short timescale stickleback have undergone rapid adaptive radiation (Rundle *et al.*, 2000), making numerous morphological and physiological adaptations to new environments. Across the radiation, freshwater and saltwater resident populations now exhibit substantial variation in traits such as body size (McKinnon *et al.*, 2004), shape (Schluter, 1993; Walker, 1997) and bony armour (Colosimo *et al.*, 2004), Figure 1.1. These adaptations are often remarkably similar across parallel environments (Cresko *et al.*, 2004; Magalhaes *et al.*, 2016; Walker, 1997) and are usually produced by the same genetic modifications (Albert *et al.*, 2008; Bowles *et al.*, 2016; Colosimo *et al.*, 2005; Schluter *et al.*, 2004), thought to be repeatedly selected

for from long-standing genetic variation in ancestral marine populations (Colosimo *et al.*, 2005).

Figure 1.1 Stickleback ecotypes found on North Uist, Scottish Western Isles. Examples of the phenotypic variation in stickleback found on North Uist. Photograph shows an adult male saltwater resident (top) and adult female anadromous (bottom) stickleback.

Stickleback are particularly interesting from an evolutionary perspective because the different ecotypes are capable of retaining largely morphologically and genetically independent entities, despite substantial opportunity for gene flow (McKinnon and Rundle, 2002). Divergent ecotypes often breed either sympatrically or parapatrically in the mouths of rivers and streams, and in accessible coastal lakes and lagoons, where hybridisation can, and frequently does, take place (Jones *et al.*, 2006; Pedersen *et al.*, 2017; Vines *et al.*, 2016). Such extensive opportunity to exchange genes should have a homogenising effect, leaving a single stickleback ecotype, yet it commonly does not (McPhail,

1992; Ravinet *et al.*, 2015; Von Hippel and Weigner, 2004). Marine and freshwater habitats differ substantially in many environmental characteristics including predation pressures (Reimchen, 1994), resource availability (Jeppesen *et al.*, 1994), water chemistry (Elser and Hassett, 1994) and flow rate (Bunn and Arthington, 2002), which are likely to favour differential adaptations in fish (Ab Ghani *et al.*, 2016; Heins *et al.*, 2016; Herbst, 2001). Ecologically based divergent selection is, therefore, strongly implicated in contributing to the maintenance of RI in stickleback inhabiting these environmental gradients (Schluter, 2001; 2009).

Whilst ecological factors undoubtedly promote divergence in stickleback, it is clear that gene flow constrains adaptive divergence in parapatric systems (Hendry *et al.*, 2002), sometimes even facilitating the collapse of previously strongly isolated pairs (Gow *et al.*, 2006; Taylor *et al.*, 2006). Stickleback were, at one point, thought to be one of the most likely examples of sympatric speciation in nature (Dieckmann and Doebeli, 1999; Mayr, 2001), but it has since become evident that while adaptive divergence often occurs between sympatric stickleback populations, it rarely progresses to complete speciation (Hendry, 2009). There are isolated exceptions where speciation appears to be in the latter stages; the most well-known examples being freshwater benthic-limnetic species-pairs in a handful of lakes in British Columbia and anadromous Pacific Ocean - Japan Sea forms in the Sea of Japan. In both cases, a period of divergence in allopatry followed by secondary contact is implicated during divergence (Higuchi and Goto, 1996; McPhail, 1993), suggesting that complete speciation in stickleback has not ever occurred entirely in sympatry (Via, 2001).

Some geographical isolation is, thus, probably necessary for the final stages of speciation to occur in stickleback (Hendry *et al.*, 2009).

MtDNA has been extremely useful for phylogenetic reconstruction in stickleback (Makinen and Merila, 2008; Taylor and McPhail, 2000; Yamada *et al.*, 2001). Orti *et al.* (1994) identified a basal Japanese and divergent North American-Atlantic clade, which was congruent with Pacific – Atlantic subdivisions identified by nuclear markers (Colosimo *et al.*, 2005; Haglund *et al.*, 1992), indicating that mtDNA in stickleback is robust for phylogenetic reconstruction. It appears that the Atlantic Ocean and all European stickleback are derived from a relatively recent colonisation (90,000 – 160,000 YBP) by Pacific ancestors (Makinen and Merila, 2008; Orti *et al.*, 1994). This Atlantic sub-group can be further divided into an ancestral Trans-Atlantic, and derived Black Sea and European lineages, which probably diverged not long after the initial Pacific colonisers arrived during the late Pleistocene, and remain, for the most part, geographically isolated from one another (Makinen and Merila, 2008). These lineages have, thus, likely experienced a long period of evolution in allopatry, raising the potential for speciation in stickleback to also be influenced by random genetic mechanisms, and mitonuclear conflict.

1.6 The study system: North Uist

North Uist is a small island (~300km^2) in the Scottish Western Isles. It is generally very low lying and comprises a mosaic of peat bogs, heathland, low hills and copious interconnected lochs and lagoons, which cover almost a third of the land surface of the island (Figure 1.2). Many of the freshwater lochs and almost all of the coastal lagoons contain stickleback, which must have colonised

the island from the sea following the last glacial retreat 12,000-16,000 YBP (Ballantyne, 2010; Jones *et al.*, 2012b), and now display a remarkable degree of phenotypic variation (Giles, 1983; MacColl *et al.*, 2013; Magalhaes *et al.*, 2016), Figure 1.1. Hybridisation between marine or anadromous ecotypes, which visit brackish coastal lagoons and some coastal freshwater habitats during the spring breeding season, and the resident salt and freshwater populations, which inhabit these locations year-round, appears to be low (El Nagar and MacColl, 2016), but the mechanisms responsible for the stark absence of hybrids in some populations have yet to be investigated. Furthermore, the stickleback of North Uist are particularly interesting from a genetic perspective as this location, along with the west coast of Scotland and Ireland (Makinen and Merila, 2008; Ravinet *et al.*, 2014a), has been identified as one of few locations in Europe in which the anciently diverged trans-Atlantic and European mitochondrial lineages meet (Rahn *et al.*, 2016). Stickleback on North Uist are, therefore, ideally situated for investigations into the processes affecting the early stages of the speciation continuum.

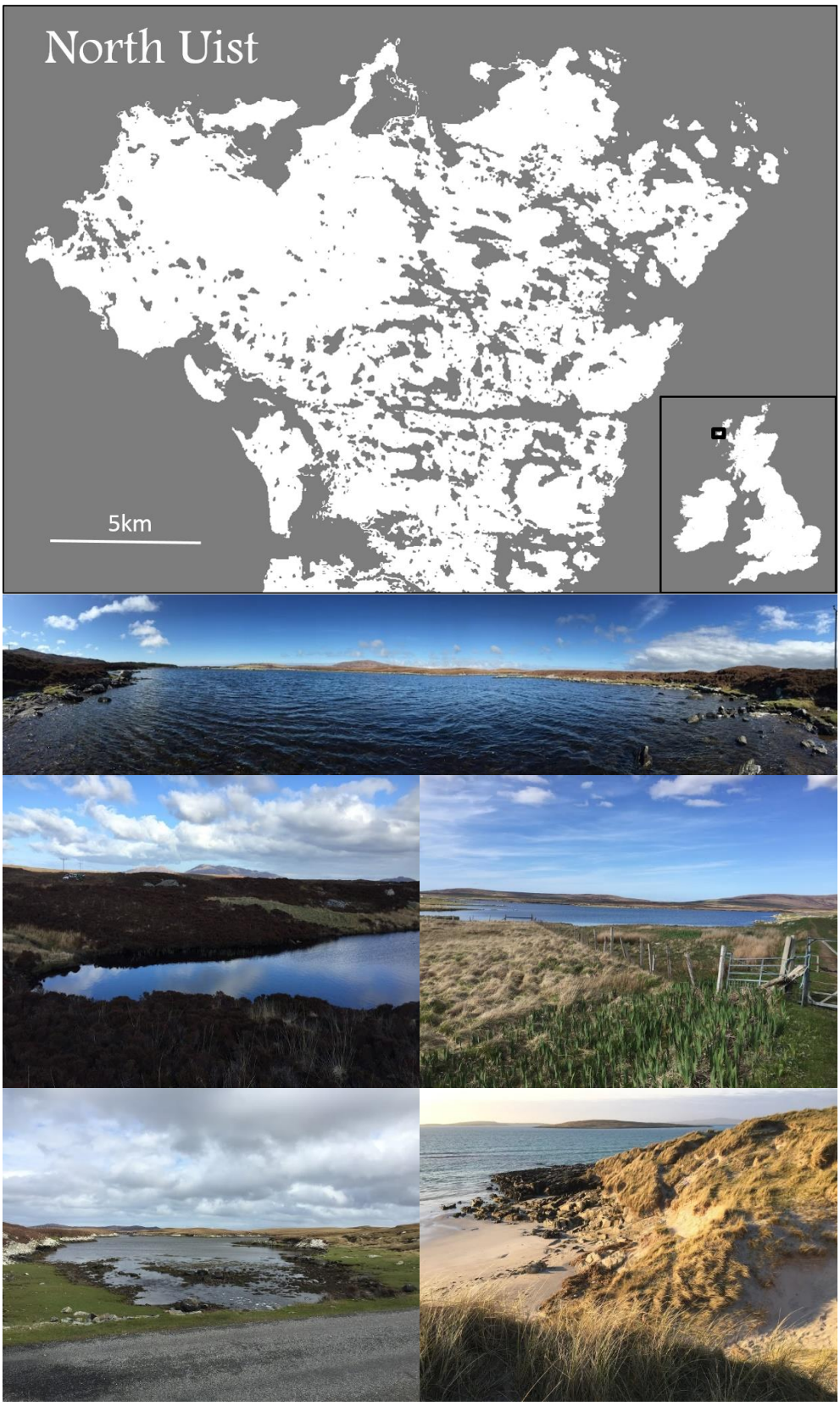

Figure 1.2 Location and topography of North Uist, Scottish Western Isles
Location of North Uist in relation to the UK and Ireland and photographs showing the local relief.

Because of North Uist's exceptionally low-lying relief (generally <50m), even relatively small changes in RSL have the potential to greatly influence the level of connectivity (or isolation) of the lochs and lagoons to the sea, making it the ideal location to study the relationship between speciation in stickleback and spatial isolation caused by fluctuations in RSL. Northern Britain has seen major fluctuations in RSL since the last glacial maximum (Shennan *et al.*, 2006a; Shennan *et al.*, 2006b; Shennan *et al.*, 2005; Shennan *et al.*, 2000), after which invasion of the newly generated freshwater habitats by marine stickleback would have become possible. However, there have been relatively few attempts to model RSL changes for the Scottish islands and reconstructions both here and for the Scottish mainland are complicated by uncertainties regarding the extent and thickness of the ice sheet over Northern Scotland, and in particular, the westerly margins of the ice sheet and its thickness over the Outer Hebrides (Kuchar *et al.*, 2012; Shennan *et al.*, 2000). Indeed, there is only one empirical RSL reconstruction directly for the Outer Hebrides (Jordan *et al.*, 2010) which, along with various models of glacio-isostatic rebound for the area (Lambeck, 1991; 1993b; c; 1995a; b; Peltier *et al.*, 2002; Shennan *et al.*, 2006a; Smith *et al.*, 2006) suggests a fluctuating rise in RSL up to present day (Figure 1.3). This is supported by the presence of extensive machair beaches along the Atlantic coast of North Uist; which were formed as a result of the shoreward transgression of offshore sand deposits following a rise in RSL (Whittington and Edwards, 1997). The specific sequence of regression and transgression found by Jordan *et al.* (2010), however, did not agree with similar reconstructions for the nearby Isle of Skye (Selby and Smith, 2007), suggesting localised factors have had a strong influence on RSL changes in the Scottish Western Isles. As such, a

detailed analysis of Holocene RSL changes in the Uists is called for if the relationship with stickleback speciation is to be properly addressed.

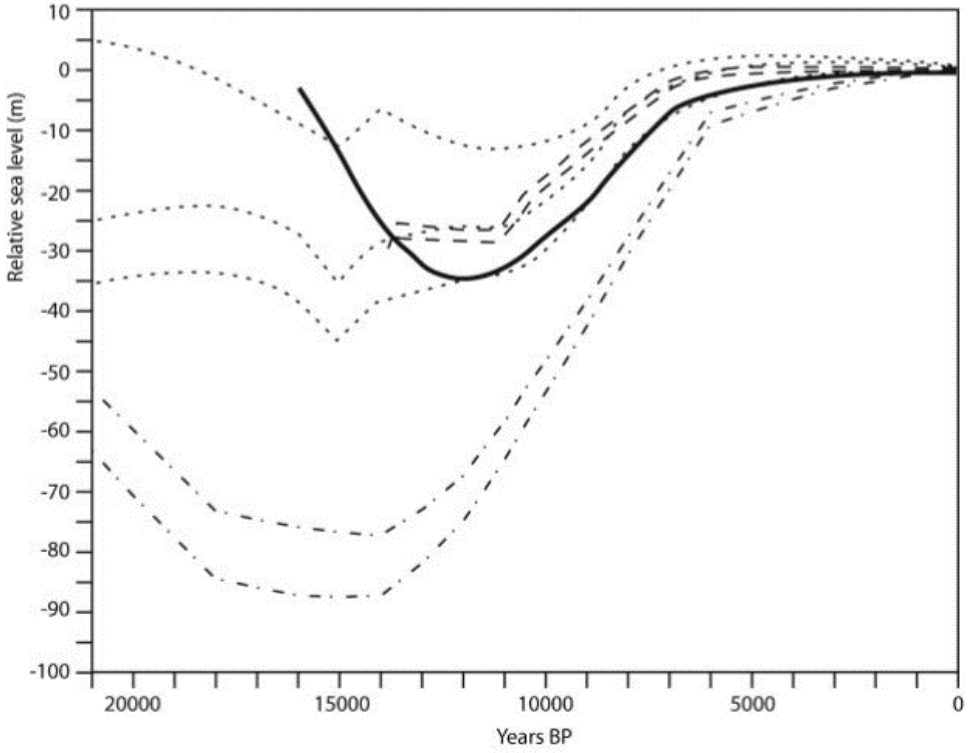

Figure 1.3 Modelled relative sea-level (RSL) change for the Outer Hebrides. RSL curves based on glacio-isostatic modelling from: ━ Lambeck (1993b), ···· Peltier *et al.* (2002), - - - Shennan *et al.* (2006) and - · - Lambeck (1995a). Graph modified from Jordan *et al.* (2010).

1.7 Thesis outline

In this thesis, I aim to address the following questions regarding the causes and contexts of speciation, using the stickleback of North Uist as a study system:

- Does the presence of multiple ecotypes within a species affect the progression of speciation? (Chapter 2)
- How important are fluctuations in spatial distributions and secondary contact for speciation? (Chapters 3 and 4)
- How important is longstanding genetic variation for speciation? (Chapter 3)
- Is reinforcement important in maintaining RI upon secondary contact? (Chapter 4)

During the course of this research, we discovered an extremely unusual and exciting phenomenon, a gravid stickleback carrying well-developed embryos within the ovaries. Chapter 5, therefore, involves a change of direction in order to capitalise on this chance finding.

Finally, I review my key findings and give suggestions as to potential avenues, opened up by this project, for further research. (Chapter 6)

CHAPTER 2 : EVOLUTIONARY MÉNAGE À TROIS – INTERFERENCE BY A THIRD ECOTYPE DESTABILIZES SPECIATION IN A THREE-SPINED STICKLEBACK SPECIES-PAIR

2.1 Abstract

Evolutionary divergence, classically viewed as bifurcation, was long thought of as a one-way street. Recently, it has become apparent that while divergence can progress to speciation, it can also collapse, and our understanding of the latter is less than it should be. To the shortlist of circumstances that favour collapse, here we add the possibility that rapid adaptive radiation may itself interfere with speciation, if the development of reinforcement between diverging pairs is interrupted by the presence of additional ecotypes. We describe a novel occurrence of well-developed species-pairs in the three-spined stickleback (*Gasterosteus aculeatus*), inhabiting multiple saltwater lagoons on the Scottish island of North Uist. Where these putative species encounter a third, normally isolated freshwater ecotype in an estuarine habitat, reproductive isolation between the species-pair collapses, and the three ecotypes form a hybrid swarm. Morphological data on body shape, armour and lateral plate phenotype, combined with genotyping for the *Eda* gene (which largely determines plate-morph) indicate that hybridisation is low or non-existent in 'two-ecotype' locations, but commonplace where all three ecotypes meet. This suggests that speciation in the three-spined stickleback model organism is destabilized in the presence of a third ecotype.

2.2 Introduction

A major goal of evolutionary ecology is to understand the role of ecological factors in promoting and constraining diversification and speciation in closely related populations. Commonly, young species pairs exhibit strong RI, with the occasional production of hybrids (Gow *et al.*, 2006). In this case a delicate balance exists between the formation of hybrids and their elimination by natural selection, and it is still possible with relative ease to move down as well as up the speciation continuum (Hendry *et al.*, 2009). If we are to comprehend speciation fully, an understanding of the mechanisms that cause the collapse of newly diverging species is equally as necessary as those which drive them to form in the first place. Competition for shared resources or for mates often plays a major role in driving movement, in either direction, along the speciation continuum (Abrams *et al.*, 2008; Drossel and McKane, 2000; Rosenzweig, 1978). On the one hand, strong competition should favour diversification into novel niches promoting opportunities for intra-population differentiation and adaptive radiation (Grant and Grant, 2006; Wong and Candolin, 2005). However, diversification can also be restricted if those niches are already occupied by competitors (Vamosi, 2003). The invasion of new competitors can even cause the collapse of newly diverging species (Bhat *et al.*, 2014; Taylor *et al.*, 2006). The complexity of such multi-species interactions during speciation is rarely studied explicitly, but is probably one of the most important factors in causing collapse.

Speciation is frequently initiated by barriers to gene flow allowing the accumulation of genetic, behavioural or temporal incompatibilities between

populations (Kozak and Wiens, 2006; Marko, 1998). Often these barriers disappear before speciation is complete, causing secondary contact between populations that are partially reproductively isolated (Barraclough and Vogler, 2000; Martin-Bravo *et al.,* 2010). If divergence has occurred to the extent that initial interbreeding produces inferior offspring, selection favours the exaggeration of existing pre-zygotic barriers to hybridisation e.g. spatial, behavioural and temporal mating characteristics (Servedio and Noor, 2003). This process, termed 'reinforcement', can complete the speciation process, following secondary contact (Dobzhansky, 1951; Hoskin *et al.*, 2005). Pre-zygotic barriers are often delicately balanced and easily altered by ecological changes, particularly competition (Taylor *et al.*, 2006), making species maintained purely by pre-zygotic barriers particularly vulnerable to collapse (Chan and Levin, 2005; Gow *et al.*, 2006). Intrinsic genetic incompatibilities usually accumulate much more slowly and thus post-zygotic barriers will only follow if reinforcement preserves the initial differentiation for a long enough time period (Seehausen *et al.*, 2014). Newly evolving sympatric species, which have had little time for post-zygotic barriers to accumulate, are thus more likely to be maintained by reinforcement of pre-zygotic barriers alone (Hoskin *et al.*, 2005), making them particularly volatile.

It does not previously seem to have been considered that rapid diversification during adaptive radiation could itself impede speciation, if additional ecotypes interfere with the development of reinforcement between any given species-pair. Studying the relationships between multiple, recently diverged ecotypes and their hybrid zones, within an adaptive radiation context, allows us to gain key insights into the role of the complex ecological role of multi-population

interactions during speciation, which would not be apparent by focusing purely on irreversibly isolated species (Barton and Hewitt, 1989; Hewitt, 1988; Jiggins and Mallet, 2000; Rieseberg *et al.*, 1999). Three-way hybrid zones are particularly useful for studying these relationships, but are generally rare in nature.

Following the Pleistocene glacial retreat, marine and sea-run anadromous three-spined stickleback (*Gasterosteus aculeatus*, hereafter 'stickleback') have repeatedly colonized freshwater and brackish coastal habitats throughout their Holarctic range (McKinnon *et al.*, 2004; Taylor and McPhail, 2000). Heritable morphological changes associated with this transition include a reduction in ectodermal bony armour, body size, and changes in body shape (Jones *et al.*, 2012b; Schluter *et al.*, 2004; Schluter and McPhail, 1992). Heavily armoured anadromous stickleback sport a 'complete' lateral plate phenotype with 31-34 plates lining each flank (Figure 2.1), and are rather morphologically uniform, with little genetic structure across large areas (Bell and Foster, 1994). Derived freshwater and saltwater resident stickleback exhibit high morphological and genetic diversity between populations, which is likely the result of multiple independent colonisations (Colosimo *et al.*, 2005). Saltwater resident (hereafter 'resident') stickleback occur year round, without migration, in shallow coastal waters: estuaries and lagoons. They generally have robust body armour with three dorsal spines and a full pelvis and pelvic spines, but a low (three to seven lateral plates) plated phenotype (Ravinet *et al.*, 2015), Figure 2.1. Anomalous stickleback, named for their greatly reduced body armour complement, are found in oligo- or dystrophic freshwater streams or isolated inland lakes (Campbell, 1979). They can exhibit complete or nearly complete loss of the pelvic structure,

dorsal spines and lateral plates, Figure 2.1. Partially-plated stickleback (with 9-29 lateral plates), can sometimes be common where other morphs hybridise (Jones *et al.*, 2006), Figure 2.1.

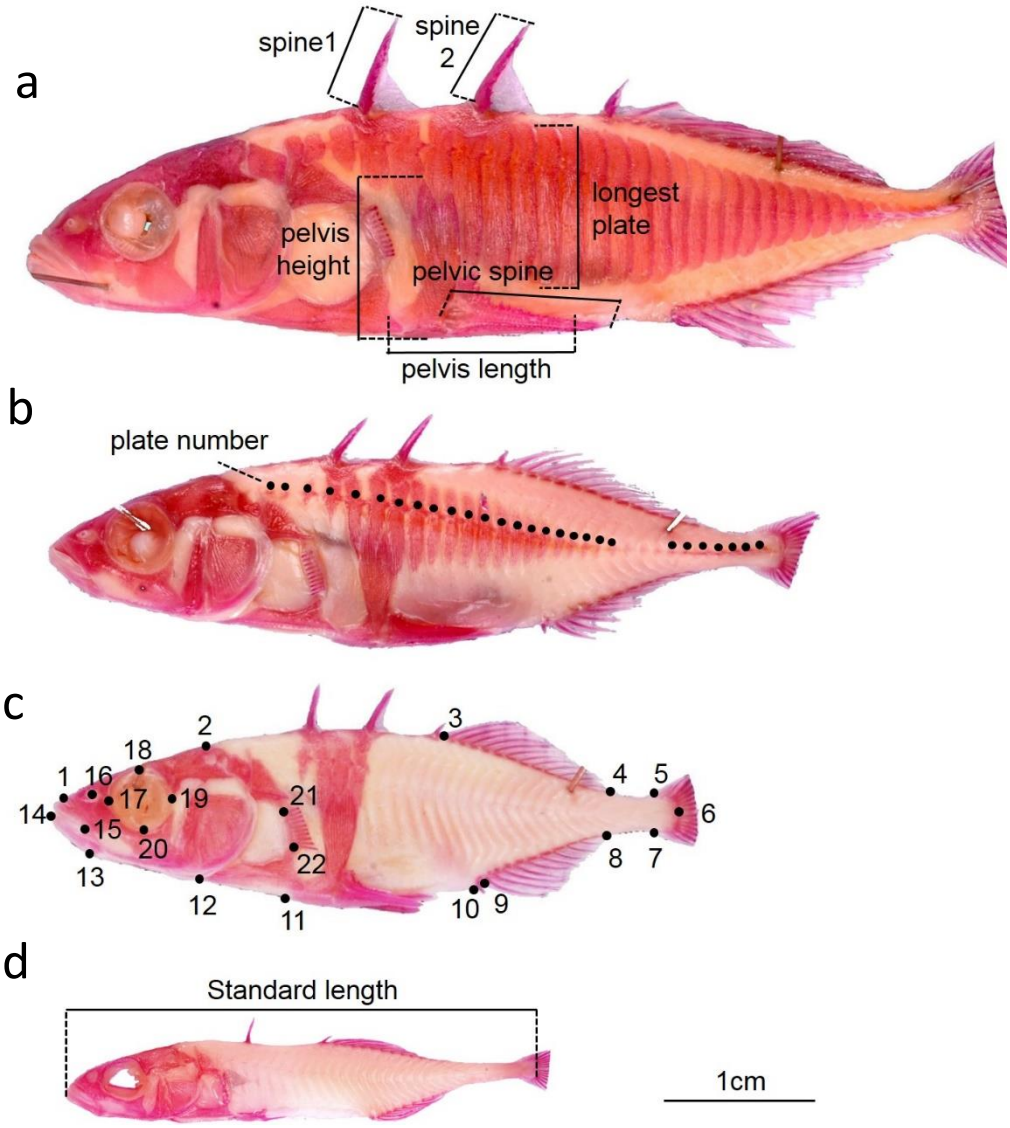

Figure 2.1 Measurements of morphological characteristics. Images of North Uist stickleback stained with alizarin red to highlight external skeletal structures. Images show examples of the different armour phenotypes on North Uist and measurements used to describe stickleback morphology: (a) anadromous ecotype from Obse; (b) partially plated hybrid individual from Cist; (c) resident ecotype from Obse and (d) anomalous ecotype from Scad. (a) and (b) show measurements of body armour, (c) shows the position of 22 landmarks used in geometric morphometric analyses and (d) shows measurements of standard length.

The evolution of some level of RI between parapatric freshwater and anadromous stickleback is common, particularly in stream environments (Hagen, 1967; Jones *et al.*, 2006; McKinnon and Rundle, 2002). Occasionally it also develops in lacustrine environments, although this is significantly less widespread (Von Hippel and Weigner, 2004). However, despite the sometimes strong RI across short distances, sympatric or parapatric habitats almost always include hybrid zones with abundant morphologically intermediate individuals (Hagen, 1967; Hay and McPhail, 2000; Heuts, 1947; Jones *et al.*, 2006). There are only a handful of cases in which hybrid, partially plated individuals are completely absent (Karve *et al.*, 2008; Ziuganov, 1995) and fewer still with direct genetic evidence for the absence of hybridisation (Drevecky *et al.*, 2013). This is likely because the divergent selection driving differential adaptations across the marine-freshwater transition is simply not strong enough for speciation to reach completion (Hendry *et al.*, 2009). Furthermore, populations that do not hybridise may be particularly vulnerable to reversal of RI because non-marine populations are mostly post-glacial in origin, and thus evolutionarily young, making it likely that RI is maintained by ecological rather than genetic factors (Schluter and Conte, 2009; Taylor *et al.*, 2006).

Reproductive isolation is well documented among other derived stickleback ecotypes, as well as between anadromous-resident populations (McPhail, 1992; Ravinet *et al.*, 2015). In the case of benthic-limnetic freshwater species-pairs, morphologically intermediate individuals are rare, comprising approximately five percent of the most diverged populations (Gow *et al.*, 2006; McPhail, 1992). Intraspecific competitive interactions have clearly played an important role in the more advanced examples of stickleback speciation (Schluter, 1995; Schluter

and McPhail, 1992; Taylor *et al.*, 2006), yet situations in which more than two ecotypes occur sympatrically are extremely rare in nature (Campbell, 1985), and only appear to have been investigated once previously (Ravinet *et al.*, 2015). Three-way contact zones have, however, been useful in furthering our understanding of speciation in other taxa (Jongejans and De Kroon, 2005; Linares, 1997; Sa-Pinto *et al.*, 2010; Tucker and Schmidly, 1981). Investigating the processes which influence the development and maintenance of RI in such evolutionarily vulnerable species complexes, particularly where more than two ecotypes co-occur can, therefore, teach us important lessons about speciation and the fragility of newly diverging species.

Here we use morphological and genetic data to identify apparently stable, strongly isolated, sympatrically breeding anadromous – resident stickleback species-pairs in multiple saline lagoons on the Scottish island of North Uist. Using data from these lagoons as 'training sets' for the morphological definition of pure ecotypes, coupled with equivalent data for anomalous fish from an isolated location, we then investigate a putative three-way hybrid zone in a coastal loch, first alluded to by Campbell (1985). We show that RI breaks down in this location, leading to extensive hybridisation between all three ecotypes, and strongly suggesting that the apparent speciation in resident-anadromous pairs is destabilized in the presence of the third anomalous ecotype. This adds to our understanding of factors which affect the speciation continuum.

2.3 Methods

2.3.1 Study site

The island of North Uist in the Scottish Western Isles, like most of the rest of Northern Europe, was likely colonized by marine stickleback following the melting of ice sheets 12,000-16,000 YBP (Colosimo *et al.*, 2005; Jones *et al.*, 2012b). Almost one third of the land surface of the island is now covered by a series of complex interconnected fresh and saline lochs and coastal lagoons and as such, is ideal for studying the marine – freshwater radiation of stickleback. The adaptive radiation of stickleback on North Uist (MacColl *et al.*, 2013; Magalhaes *et al.*, 2016) harbours three putatively isolated stickleback ecotypes; anadromous, saltwater resident and freshwater anomalous co-occurring in a single location (Campbell, 1979), as well as numerous examples of resident-anadromous sympatry, making it a useful location to study inter-ecotype interactions and their effect on RI. In addition 'normal' monotypic, low-plated freshwater populations are also found in many isolated lochs (MacColl *et al.*, 2013; Magalhaes *et al.*, 2016), but are not considered here.

We first investigated the migratory range of anadromous stickleback on North Uist to constrain the extent of overlap between ecotypes. We then characterized morphological and genetic separation between previously undescribed sympatric resident and anadromous species-pairs in six coastal lagoons on North Uist (Figure 2.2, Table 2.1), and compared them to the highly diverged inland anomalous ecotype (sampled from Scad, Figure 2.2 and Table 2.1). Finally we investigated the distribution, morphology and genetics of stickleback in Cist (Figure 2.2, Table 2.1), a loch which has previously been identified as potentially

containing three sympatric stickleback ecotypes; an extremely rare phenomenon in nature (Campbell, 1985). Cist is a small, tidally influenced coastal loch, which is predominantly freshwater for most of the lunar cycle, although it is readily accessible to anadromous fish at spring tides. The largest freshwater catchment on North Uist drains through Cist to the sea (Figure 2.2). Much of this catchment, including one of our sampling locations, Scad, is inhabited by highly derived freshwater anomalous stickleback (MacColl *et al.*, 2013; Magalhaes *et al.*, 2016). The flow of water through the catchment means that anomalous fish are washed downstream into Cist while migration in the opposite direction is made difficult.

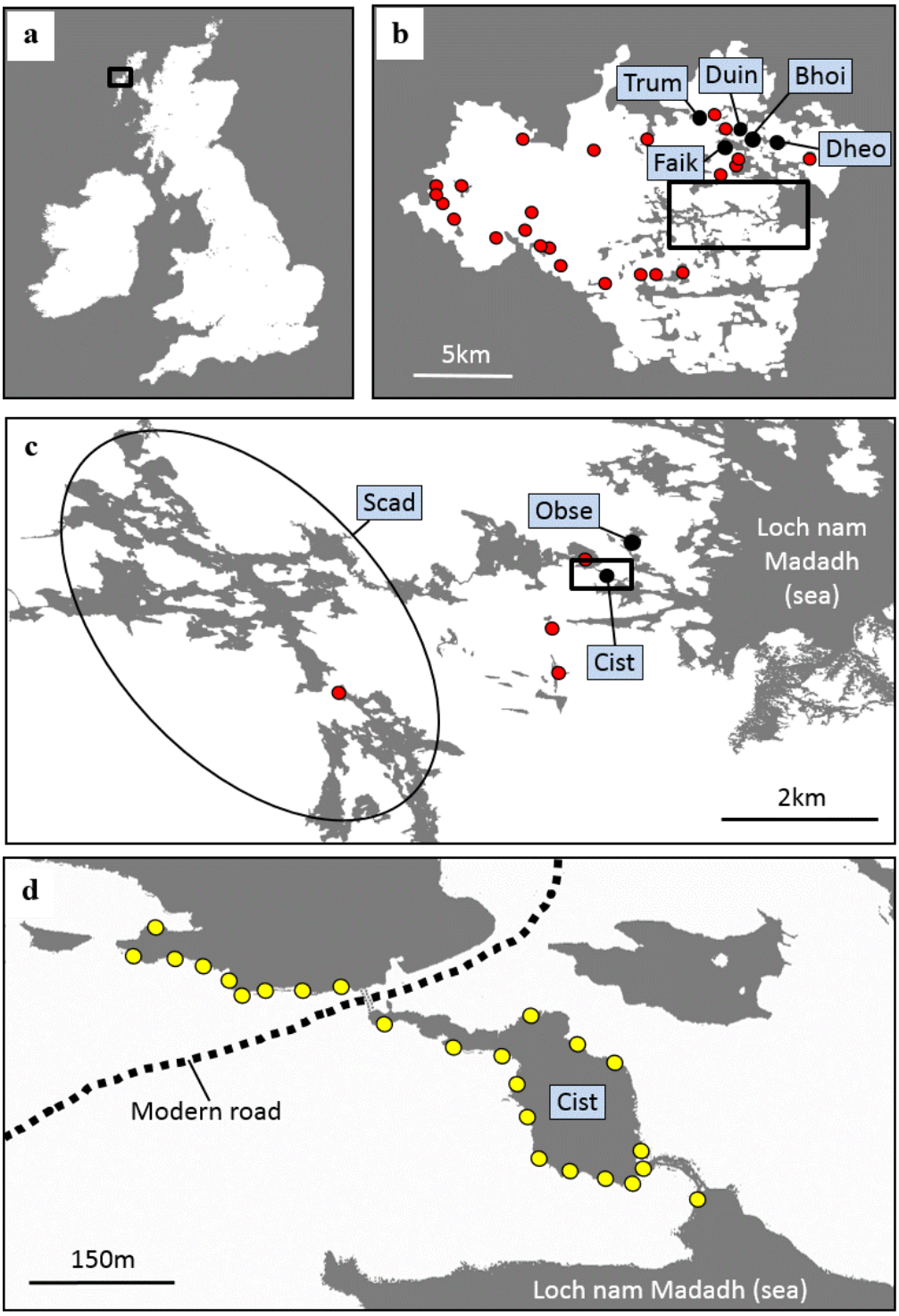

Figure 2.2 Sampling locations. Maps showing (a) the location of North Uist relative to the UK and Ireland, (b & c) the location of sampling sites from which stickleback were collected for morphological and / or genetic analyses (black circles) and locations sampled to determine anadromous migratory ranges (red and black circles) and (d) locations of sample sites within Cist (yellow circles).

Table 2.1 Description of sample sites. Loch locations are given by latitude followed by longitude. Sampling years in which anadromous occurrence was recorded, and (^{s}) in which stickleback were taken for morphological and/or genetic analysis. Sample sizes (N) are shown for morphological and genetic (curved parentheses) analyses. Ecotypes present describes the three pure ecotypes: anomalous, resident and anadromous pictured in Figure 2.1, and in locations where freshwater fish were not characterised for armour loss they are described as 'freshwater'. Conductivity describes absolute conductivity, distance from the sea describes approximate shortest distances through water estimated using satellite imagery (GoogleEarth, version 7.1.8.3036).

Loch ID	Location	Sampling year	N	Ecotypes present	Distance from the sea (km)	Salinity	Conductivity (µS/-cm)
Scad	57°35′6"N; 7°14′10"W	2007, 2008, 2010, 2011, 2013^{s}, 2015, 2017	18	anomalous	7.499	freshwater	147
Faik	57°38'7"N; 7°12'54"W	2007, 2008, 2009, 2010, 2011, 2015^{s}, 2016, 2017	39 (24)	resident, anadromous	0.027	brackish	35,943
Obse	57°36'6"N; 7°10'22"W	2008, 2009, 2010, 2011, 2013, 2015^{s}, 2016, 2017	39 (20)	resident, anadromous	0.193	brackish	38,470
Duin	57°38'35"N; 7°12'40"W	2011, 2013, 2014, 2015^{s}	39 (9)	resident, anadromous	0.021	brackish	27,460
Trum	57°39'9"N; 7°14'35"W	2015^{s}	40 (11)	resident, anadromous	0.015	brackish	36,288
Bhoi	57°38'37"N; 7°12'6"W	2015^{s}	51	resident, anadromous	0.000	marine	47,515
Dheo	57°38'26"N; 7° 9'58"W	2015^{s}	34 (2)	resident, anadromous	0.000	marine	42,720
Cist	57°35'55"N; 7°10'39"W	2015^{s}, 2016	190 (68)	anomalous, resident, anadromous	0.352	freshwater	196

Loch ID	Location	Sampling year	N	Ecotypes present	Distance from the sea (km)	Salinity	Conductivity (µS/-cm)
Dubh	57°34′54"N; 7°24′12"W	2007, 2008, 2010, 2011, 2013	N/A	freshwater	1.48	freshwater	174
Host	57°37′40"N; 7°29′18"W	2007, 2008, 2010, 2011, 2013, 2014, 2015	N/A	freshwater, anadromous	1.00	freshwater	340
Fhai	57°34'6"N; 7°22'45"W	2010, 2014	N/A	freshwater, anadromous	0.72	freshwater	142
Nage	57°38'34"N; 7°25'16"W	2010, 2013	N/A	freshwater	0.72	freshwater	277
Tros	57°35'3"N; 7°24'45"W	2011	N/A	freshwater, anadromous	0.63	freshwater	173
Grog	57°36′54"N; 7°30′40"W	2011, 2014, 2015, 2016	N/A	freshwater, anadromous	0.47	freshwater	342
Sgea	57°36'1"N; 7°10'56"W	2015, 2016	N/A	anomalous, resident, anadromous	0.44	freshwater	188
Iala	57°37'54"N: 7°12'31"W	2009, 2011, 2013, 2014, 2015	N/A	freshwater, anadromous	0.30	freshwater	185
StrmS	57°36'41"N; 7°12'12"W	2015	N/A	anomalous, resident, anadromous	0.15	freshwater	572
Strm	57°36'36"N; 7°11'1"W	2015	N/A	resident, anadromous	0.04	brackish	20,882

Loch ID	Location	Sampling year	N	Ecotypes present	Distance from the sea (km)	Salinity	Conductivity (µS/-cm)
Caig	57°37'59"N; 7° 6'37"W	2015, 2016	N/A	freshwater, anadromous	0.13	freshwater	389
Mora	57°34'29"N; 7°16'31"W	2007, 2008, 2010, 2011, 2013	N/A	freshwater	10.00	freshwater	173
Eisi	57°37'50"N; 7°21'8"W	2010, 2011, 2013	N/A	freshwater	7.09	freshwater	130
Gill	57°36'4"N; °24'37"W	2007, 2008, 2010, 2011, 2013, 2014	N/A	freshwater	5.03	freshwater	159
Daim	57°35'32"N; 7°12'30"W	2007, 2008, 2010, 2011, 2013	N/A	freshwater	0.27	freshwater	162
Maga	57°36′10"N; 7°28′54"W	2007, 2008, 2010, 2011, 2013, 2014	N/A	freshwater	3.06	freshwater	262
Fada	57°37'3"N; 7°12'45"W	2013, 2014, 2015	N/A	freshwater	3.00	freshwater	136
Aroi	57°35'40"N; 7°25'52"W	2007, 2008, 2010, 2011, 2013, 2014	N/A	freshwater	2.57	freshwater	173
Sann	57°35′12"N; 7°27′48"W	2010	N/A	freshwater	2.49	freshwater	375
Torm	57°33'46"N; 7°19'1"W	2007, 2008, 2010, 2011, 2013, 2014, 2015	N/A	freshwater	1.72	freshwater	181
Bhar	57°34'16"N; 7°18'8"W	2007, 2008, 2010, 2011, 2013, 2014, 2015	N/A	freshwater	1.53	freshwater	140
Feit	57°36'25"N; 7°30'17"W	2010	N/A	freshwater	1.36	freshwater	490

Loch ID	Location	Sampling year	N	Ecotypes present	Distance from the sea (km)	Salinity	Conductivity (µS/-cm)
Crei	57°38'41"N; 7°13'33"W	2009, 2011, 2013	N/A	freshwater	1.34	freshwater	195
Stru	57°33'28"N; 7°20'58"W	2011, 2014	N/A	freshwater	1.30	freshwater	165
Chru	57°35'37"N; 7°11'44"W	2008, 2009, 2010, 2011, 2013, 2014, 2015	N/A	freshwater	0.66	freshwater	149
Aong	57°38'46"N; 7°16'16"W	2010, 2014	N/A	freshwater	0.49	freshwater	218
Buai	57°38′48"N; 7°11′48"W	2007, 2008, 2011, 2014, 2015	N/A	freshwater	0.49	freshwater	210
Geir	57°38′34"N; 7°25′18"W	2010, 2015	N/A	freshwater	0.42	freshwater	131
Reiv	57°36'39"N; 7°30'50"W	2008, 2009, 2010, 2011, 2013, 2014, 2015, 2016, 2017	N/A	freshwater	0.10	freshwater	433

2.3.2 Sample collection

Stickleback were caught using eight to thirty unbaited minnow traps (Gee traps, Dynamic Aqua, Vancouver) per loch set overnight in water approximately 30-100cm deep. Captured sampled stickleback were either assessed for morphological characteristics and released, or if required for morphological and/or genetic analysis, immediately euthanized with an overdose of tricaine methanesulfonate or 'MS222' (400 mg L^{-1}), and killed by destruction of the brain, in accordance with Schedule One of UK Home Office regulations. The caudal fin and both pectoral fins were removed from dead fish and stored in 100% ethanol at -20°C for genetic analyses. Finally, fish were preserved in 70% ethanol until morphometric analysis approximately one month later.

2.3.2.1 *Anadromous ranges*

To assess anadromous occurrence; 37 lochs (Figure 2.2) known to contain stickleback (MacColl *et al.*, 2013; Magalhaes *et al.*, 2016) were trapped at least once between March and June of 2007-2011 and/or 2013-2017 (Table 2.1) and the occurrence of anadromous stickleback recorded. Stickleback were subsequently released unless required for other analyses. Approximate shortest distances from the sea (through water) were estimated for each loch using satellite imagery (GoogleEarth, version 7.1.8.3036) and salinity measurements (Table 2.1) were taken using a conductivity meter at the time of stickleback sampling.

2.3.2.2 *Investigation of species-pairs*

To investigate differentiation in anadromous-resident stickleback species-pairs 14-20 individuals per ecotype, per loch were selected between April and May of 2013 and 2015, from six coastal lagoons: Faik, Obse, Duin, Trum, Bhoi, and Dheo (Figure 2.2, Table 2.1), along with all morphologically intermediate individuals (this was only one individual across the six species-pair locations). To quantify the extent of morphological differences between anadromous and resident fish, and the anomalous ecotype, a sample of the latter were also collected from a pure population in Scad (n=18) between April and June 2013.

2.3.2.3 *Characterization of tri-ecotype hybrid zone*

To investigate potential sympatry between three stickleback ecotypes in Cist, traps were set daily for a five day period in late April 2015 at 20 locations along a ca. 600m portion of the loch shore (Figure 2.2). All stickleback sampled from Cist (n=190) were retained for morphological and genetic analyses and the location of each sample site was recorded using a handheld GPS unit (Garmin GPSmap 60CSx).

2.3.3 Comparison of ecotypes

To compare morphological and genetic characteristics of stickleback in Cist with the three putative 'pure' stickleback ecotypes on North Uist, we created a training set to quantify the usual characteristics of each ecotype, where they exist as largely reproductively isolated populations. Stickleback in Scad are reproductively isolated from all other ecotypes on North Uist as a result of their allopatric inland location (Figure 2.2) and therefore were selected to represent

the anomalous ecotype in the training set. Resident and anadromous stickleback do not exist in isolation on North Uist, and for this reason, these ecotypes were characterized in the training set by pooling morphological and genetic data (n=149 and n=93 respectively) from six coastal lagoons in which resident and anadromous ecotypes are present but phenotypically intermediate individuals are totally or almost totally absent (Faik, Obse, Duin, Trum, Dheo and Bhoi, Figure 2.2, Table 2.1). Morphological and genetic data from the training set were then compared to that of stickleback from Cist. Where classification of ecotypes in Cist was required, a discriminant function was created using the training set, and all Cist individuals that fell inside one of the 95% confidence ellipses for the three 'pure' training set ecotypes were categorized as those respective ecotypes. Individuals which fell outside the confidence ellipses were classified as either resident-anadromous, resident-anomalous or anadromous-anomalous hybrids according to their two closest 'pure' ellipses.

2.3.4 Morphological analysis

To visualize external bony 'armour' structures all individuals from the training set (n=260) and Cist (n=190) were bleached and stained with alizarin red following a standard procedure (Peichel *et al.*, 2001). The left side of each stickleback was photographed using a tripod mounted digital SLR camera fitted with a macro lens and macro digital ring flash. Images were scaled and measurements of standard length, first and second dorsal spine length (from first point of insertion to tip of the spine), longest plate length, pelvis height, pelvis length, pelvic spine length, and number of lateral plates were taken to the nearest 0.01mm using ImageJ, version 1.48v (Schneider *et al.*, 2012), Figure 2.1.

To analyse body shape, images were re-scaled and 22 landmarks, based on configurations which have previously been shown to sufficiently describe stickleback morphometrics (Walker and Bell, 2000), were placed on each image using tpsDig, version 2.16 (Rohlf, 2010), Figure 2.1. Centroid size, used as an estimate of overall body size, was calculated using the same 22 landmarks (Figure 2.1) in the program MorphoJ, version 1.06d (Klingenberg, 2011). Measurements of standard length (from the tip of the snout to the end of the caudal peduncle), Figure 2.1, were also taken for the standardization of body armour variables.

2.3.5 Eda genotyping

The *Ectodysplasin A* (*Eda*) genotype, which plays a key role in determining plate morph (Colosimo *et al.*, 2005; Colosimo *et al.*, 2004), of a subset of sampled fish in resident-anadromous species-pairs and Cist was determined as a simple assay of the occurrence of hybrids. There are two key *Eda* alleles; Eda^{L} (low) and Eda^{C} (complete), which are associated with low and completely plated phenotypes respectively. Heterozygous, hybrid individuals usually, but not always (Cresko *et al.*, 2004; Lucek *et al.*, 2012), exhibit a partially plated phenotype (Colosimo *et al.*, 2005). Because lateral plate morph is likely to be the key distinguishing factor between ecotypes (Ravinet *et al.*, 2015) we used *Eda* genotyping to ensure that lateral plate counts accurately reflected the prevalence of adult resident – anadromous hybrids.

Genomic DNA was extracted from fin clips for 33 resident, 32 anadromous and the single morphologically intermediate stickleback across five of the six species-pair lochs and 68 individuals from Cist using either Qiagen DNeasy

blood and tissue kits, Quanta Biosciences Extracta™ DNA prep for PCR-Tissue kits or following a proteinase K and ethanol precipitation procedure (Goldenberger *et al.,* 1995). DNA was amplified for the *Stn382* microsatellite marker, which flanks a 60bp indel in the first intron of *Eda*, using the primers: forward 5' CCCTTAGAGAATTTCCTAGCAG 3', reverse 5' CTTGTCCCGGATCATACGC 3', taken from (Colosimo *et al.,* 2005). Polymerase chain reaction (PCR) was carried out in 20µl reaction volumes consisting of 8µl nuclease-free H_20, 9µL 2X Biomix™ red reaction mix (Bioline), 1µl of forward and reverse primers (10µM) and 1µl of template DNA (approximately 20ng). Thermocycling was carried out as follows: Initial denaturation at 98°C for two minutes, followed by 35 cycles of denaturation at 98°C for 15 seconds, annealing at 58°C for 15 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for five minutes. *Stn382* produces either a 158bp product, associated with the Eda^L allele or a 218bp product associated with the Eda^C allele (Colosimo *et al.*, 2005). PCR products were analysed on a 1.5% agarose gel in TE buffer at 110V for 50 minutes and product size was determined by visual comparison with 100bp ladder.

2.3.6 Statistical analysis

Unless otherwise stated all analyses were carried out using R, version 3.4.1 (R.Core.Team, 2017). Where generalized linear models (GLMs) were used model simplification was conducted using a stepwise, top down approach with likelihood ratio tests to compare models and the least significant terms removed first. The goodness-of-fit of the best fitting model was then evaluated using residual and Quantile-Quantile (Q-Q) plots and Akaike information criterion

(AIC), and models were transformed and re-fitted if the necessary family criteria were violated. The statistical significance of predictor variables in the final models were determined using likelihood ratio tests.

The relationship between anadromous occurrence and distance from the sea was analysed using a binomial logistic regression and logit link function with anadromous occurrence (presence/absence) as the response variable and distance from the sea, the potentially confounding variable of salinity, and the interaction between the two, as predictor variables.

Landmark data were analysed using MorphoJ, version 1.06d (Klingenberg, 2011). A Procrustes fit, aligning specimens by their main axis, was performed to remove size and rotation biases. A multivariate partial least squares regression analysis, with log centroid size as an independent variable, was used to remove allometric variation in body shape (Reist, 1986). Regression residuals were then exported into R where they were standardised and scaled, and variation in body shape for all individuals was visualized using a principal components analysis (PCA), implemented by singular value decomposition.

All measured elements of armour, excluding plate number, which was independent of body size in our data set, were size-standardized by taking the residuals of a regression against standard length. To visualize the axis of greatest variation in bony armour, a PCA was carried out using the singular value decomposition method with scaling on the armour regression residuals and plate count data.

The effect of *Eda* genotype on plate morph was assessed using a chi-squared test with ‘complete’, ‘partial’ and ‘low’ plated categories for plate morph and ‘CC’,

'CL' or 'LL' genotypes. The proportion of variance in plate number that was explained by *Eda* genotype was calculated for all individuals and for the training set and Cist separately using McFadden's pseudo R^2 (McFadden, 1973). Between-group differences in the proportions of variance explained were then calculated using a Mann-Whitney U test.

A linear discriminant analysis (LDA) was performed to determine the extent of phenotypic separation between anomalous, resident and anadromous stickleback in the training set and to compare that separation to Cist. For simplicity the single morphologically and genetically intermediate individual from Faik was removed. All armour and body shape regression residuals from the training set along with plate number and centroid size were scaled and used as predictor variables, with 'anomalous', 'resident' and 'anadromous' categories as grouping variable classes. The accuracy of the LDA in predicting group membership of individuals within the training set correctly was determined using a jack-knifed, leave-one-out cross validation approach. The LDA was subsequently used to predict where all stickleback from Cist would fall in phenotypic space relative to the training set. To determine the accuracy of the LDA in assigning individuals from Cist to each group a k-means clustering analysis was used with the Dunn index (the ratio of the smallest inter-cluster distance to the largest intra-cluster distance between observations) as cluster validation. The LDA was performed using the MASS package version 7.3-45 (Venables and Ripley, 2002) and cluster analyses were performed using the packages mclust version 5.2 (Fraley and Raftery, 2002; Fraley *et al.*, 2012) and clValid version 0.6-6 (Brock *et al.*, 2008) in R.

Differences in centroid size between ecotypes in the training set and Cist were analysed using t-tests. Welch's t-tests were used to account for unequal variances and sample sizes between groups. To make this comparison, individuals from Cist were classified into one of three ecotype categories based on the classifications produced by the LDA and all Cist individuals falling outside of these categories were excluded from body size comparisons.

To analyse the relationship between salt and freshwater ecotypes along the Cist transect, all individuals classified as 'hybrids', based on the LDA, were excluded (leaving n=104) and a binomial GLM and logit link function was used. The number of saltwater ecotypes (resident and anadromous) as a proportion of the total number of pure ecotypes caught (resident, anadromous and anomalous) was used as the response variable with distance from the sea as the predictor variable.

To analyse the distribution of individuals across the three ecotype and two hybrid categories (resident-anadromous and resident-anomalous, no fish fell intermediately between anadromous and anomalous ecotypes) in Cist a negative binomial GLM with logit link function was used with number of fish caught as the response variable and ecotype and distance from the sea as predictor variables.

2.4 Results

2.4.1 Existence of resident-anadromous species-pairs

2.4.1.1 *Anadromous occurrence*

Across all trapping data, the occurrence of anadromous fish on North Uist declined sharply within just a few hundred meters of the sea (binomial GLM: $LR_1 = 27.16$, $p < 0.001$, Figure 2.3). No anadromous fish were caught further than ~1km from the sea through water, although freshwater fish are found up to 10km inland. Neither salinity, nor the interaction between distance and salinity were significant in our model.

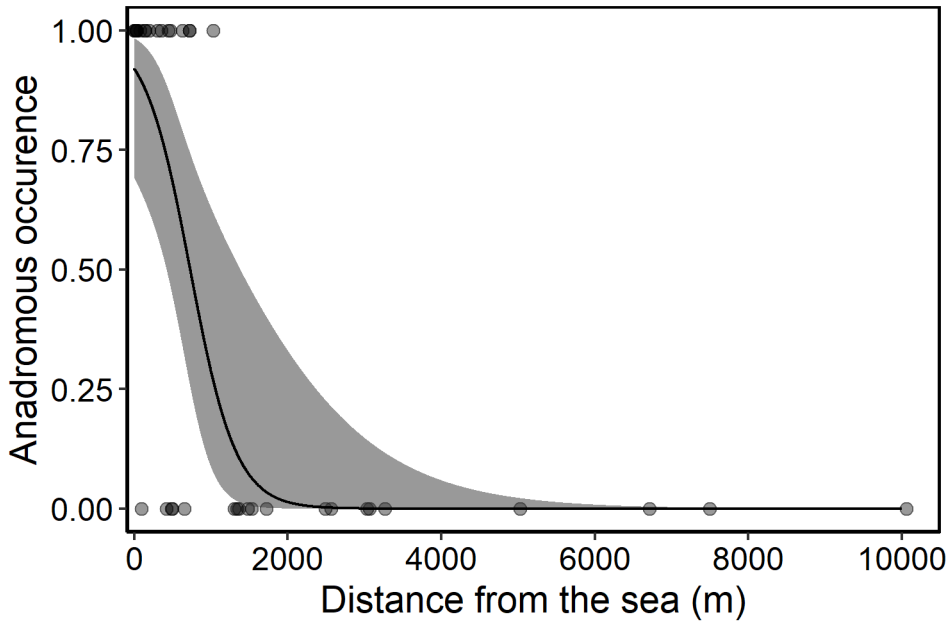

Figure 2.3 Anadromous occurrence. The occurrence of anadromous stickleback was strongly associated with distance from the sea. The graph shows the presence / absence of anadromous stickleback (grey circles) for 37 lochs on North Uist (see Figure 2.2 and Table 2.1 for sampling locations) and the fit of a binomial generalized linear model + logit link function (solid line) with anadromous occurrence as the dependent variable and distance from the sea as the predictor variable. The associated standard errors are depicted by the grey ribbon.

2.4.1.2 *Species-pair morphological separation*

The first body shape principal component (shape PC1) accounted for 24% of total variation and largely described a shift from deep bodied, laterally compressed forms to a shallower bodied, more elongated profile, with a more forward positioning of the pelvis and dorsal spines, and elongate caudal peduncle (Figure 2.4). The second principal component (shape PC2) explained 18% of variation in body shape and described changes in the positioning of the pectoral fins, snout and anal spine (Figure 2.4). Resident and anadromous ecotypes from the species-pair lagoons were largely similar in body shape, with the resident 95% confidence ellipse falling almost entirely inside that of the anadromous fish. Residents, however, were considerably less variable in body shape than anadromous fish and clustered at the upper extreme of shape PC1.

Anadromous fish were clearly larger than residents (average centroid size: anadromous, 111.4 ± 11.0 a.u.; residents, 63.8 ± 10.4 a.u., Figure 2.5).

Armour PC1 (explaining 76% of variation) described an increase in the size of all measured armour elements with length of the pelvic spine, overall size of the pelvis and length of the longest plate having the highest loadings. Armour PC2 (explaining 11% of variation in the data) described an increase in lateral plate number, and was weakly negatively correlated with pelvis size (Figure 2.6). Resident and anadromous populations from species-pair lagoons showed no separation along armour PC1, but were clearly separated along armour PC2, indicating that the number of lateral plates is the key armour element distinguishing resident from anadromous fish. A single individual from Faik with a partially plated phenotype (termed a hybrid for the purposes of this

analysis) fell closest to the anadromous population as a result of its lateral plate count (Figure 2.6). In terms of plate morph, resident fish were all low-plated and had 3-7 lateral plates with a mean of 4.8 ± 0.07, and anadromous fish were completely plated with 31-34 lateral plates and had a mean of 32.6 ± 0.07. The single morphologically intermediate individual from Faik was partially plated with 25 lateral plates.

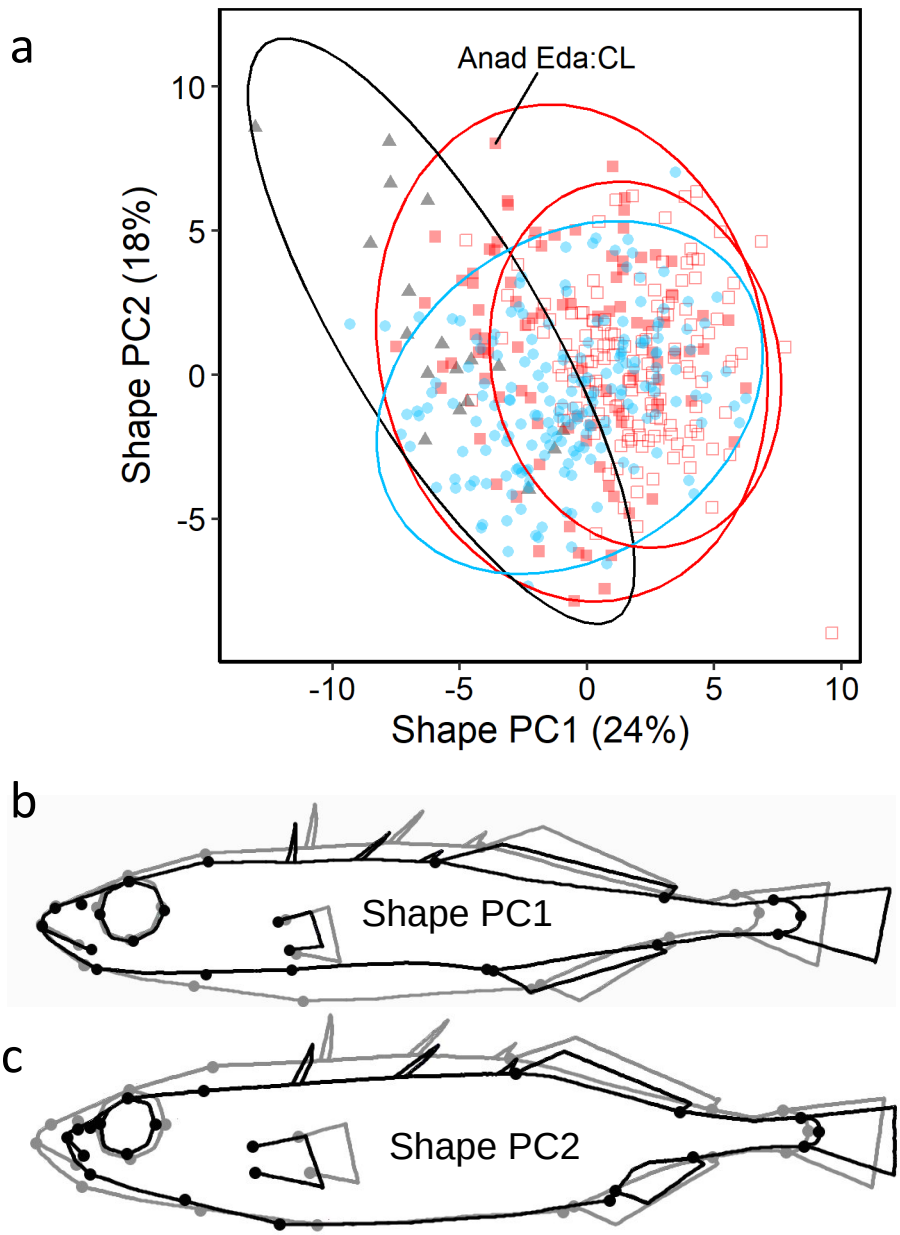

Figure 2.4 Variation in body shape among North Uist stickleback. (a) Distribution of phenotypes and their associated 95% confidence ellipses across the first two principal components of 44 body shape variables (derived from 22 landmarks). Grey triangles indicate individuals from Scad, an isolated loch containing a single anomalous freshwater ecotype; red shapes indicate fish from six pooled species-pair lagoons with resident ecotypes represented by open squares, anadromous by closed squares and the single phenotypic hybrid individual from Faik by the plus. Blue circles indicate stickleback from Cist, in which all three ecotypes occur sympatrically. The single phenotypically anadromous individual from Trum with a heterozygous Eda genotype is labelled 'Anad Eda:CL'. Shape changes represented by PCs 1 and 2 are shown in warped outline drawings (b) and (c) respectively.

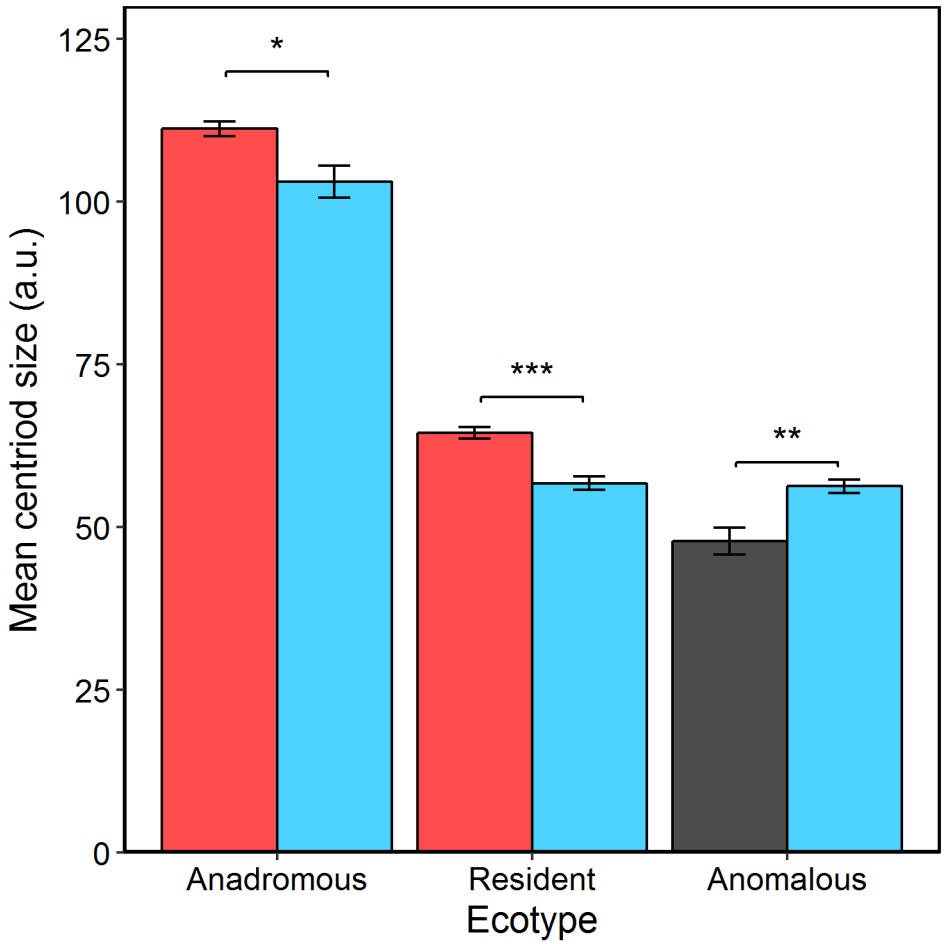

Figure 2.5 Variation in body size among North Uist stickleback. Differences in centroid size between the training set (species-pairs; red bars, and Scad; grey bar) and Cist (blue bars). Error bars represent the standard error of the mean (*SEM*) and stars indicate the significance (* = <0.05, ** = <0.01, *** = <0.001) of Welch's t-tests between groups represented by black brackets.

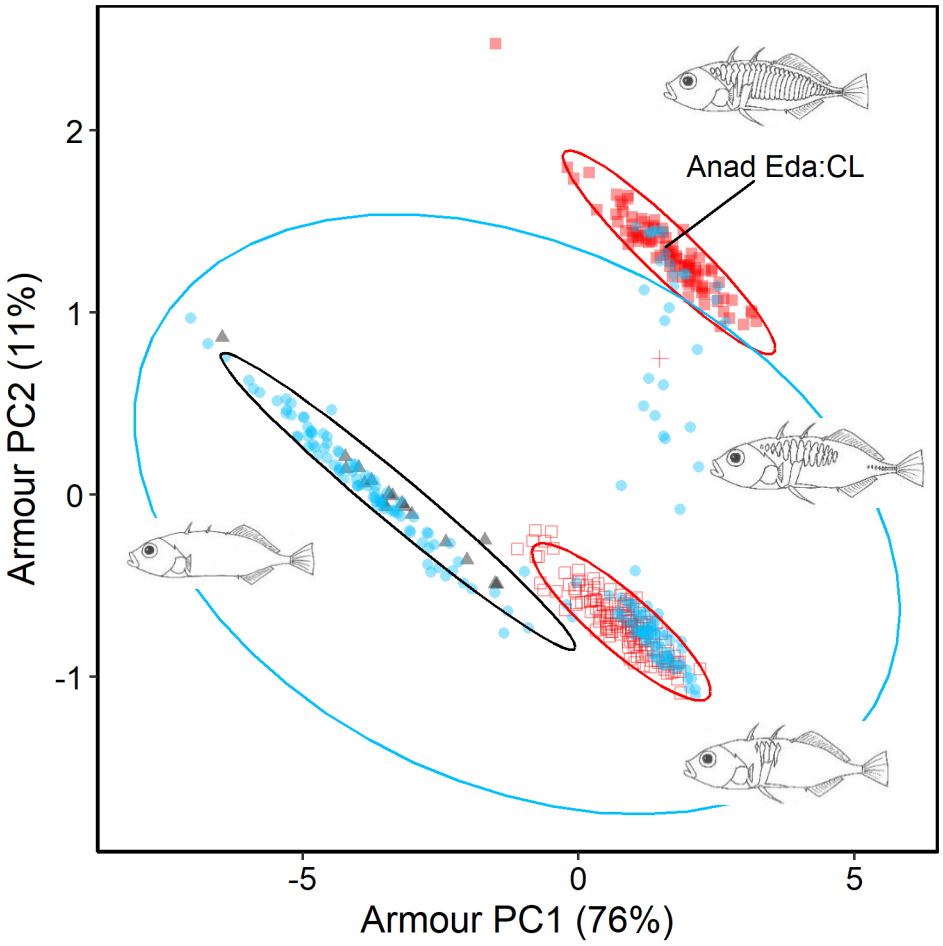

Figure 2.6 Variation in body armour among North Uist stickleback. Distribution of armour phenotypes and their associated 95% confidence ellipses across the first two principal components on seven body armour variables (see Figure 2.1). Armour PC1 describes an increase in the relative size of all continuous measured armour elements and armour PC2 largely describes an increase in the number of lateral plates and a decrease in pelvis size. Grey triangles indicate individuals from Scad, an isolated loch containing a single anomalous freshwater ecotype, red shapes indicate fish from six pooled species-pair lagoons with resident ecotypes represented by open squares, anadromous by closed squares and the single phenotypic hybrid individual from Faik by the plus symbol. Blue circles indicate stickleback from Cist, in which all three ecotypes occur sympatrically. The single phenotypically anadromous individual from Trum with a heterozygous Eda genotype is labelled 'Anad Eda:CL'. Illustrations show the associated threespine stickleback armour phenotypes.

2.4.1.3 *Species-pair genetic separation*

Eda genotype was strongly associated with plate morph (X^2 = 200.39, *df* = 6, *p* < 0.001) and accounted for 85% of variation in plate number across 134 genotyped North Uist individuals. Resident fish from species-pair lagoons (n=33) were fixed for the Eda^L allele and anadromous fish were approaching fixation for the Eda^C allele with only one out of the 32 genotyped individuals possessing a heterozygous, rather than homozygous CC genotype (Table 2.2). The only partially plated individual caught across all species-pair lagoons was also an *Eda* heterozygote (Table 2.2).

Table 2.2 *Eda* genotypes vs lateral plate phenotypes. Comparison of phenotypes vs. genotypes for stickleback from five pooled species-pair lochs (n=66) and Cist (n=68). Low (anom) refers to stickleback with an anomalous - low plated phenotype with 0-2 lateral bony plates, Low (res) refers to stickleback with a resident low plated phenotype with 3-7 lateral plates, Partial refers to fish with 9-29 lateral plates and Comp refers to fish with 31-33 lateral plates. CC represents fish with two Eda^C alleles, LL: two Eda^L alleles and CL: one Eda^C and one Eda^L allele.

Plate morph species-pairs				**Plate morph Cist**				
	Low (res)	Partial	Comp		Low (anom)	Low (res)	Partial	Comp
CC	0	0	31	CC	0	0	1	8
CL	0	1	1	CL	0	3	13	3
LL	33	0	0	LL	24	16	0	0

2.4.2 Morphology of isolated anomalous stickleback

'Pure' anomalous fish from Scad clustered at the lower end of shape PC1; indicating a very slender, elongated profile, but showed no separation from the other ecotypes along shape PC2. Anomalous 95% confidence ellipses in the

body shape analysis were overlapping with both resident and anadromous populations, although there was considerably more overlap with the anadromous fish (Figure 2.4). Anomalous fish were also considerably smaller than both anadromous and resident fish from species-pair lagoons with a mean centroid size of 47.9 ± 8.6 a.u. (Figure 2.5).

Differentiation between anomalous fish and the other ecotypes was most apparent in the body armour PCA, with anomalous fish scoring much lower on armour PC1, as a result of their significantly reduced armour complement (Figure 2.6). Anomalous fish were also separated from the other ecotypes along armour PC2, falling intermediately between resident and anadromous ecotypes as a result of their low plate number and complete lack of pelvic structure. In terms of plate morph, pure anomalous stickleback were all low-plated morphs with 0-2 and a mean of 0.2 ± 0.12 lateral plates per fish.

2.4.3 Breakdown of morphological and genetic separation in Cist

2.4.3.1 *Morphological destabilization in Cist*

Cist fish largely encompassed all of the body shapes found in the training set along shape PC1. Shape PC2 did not generally separate populations or ecotypes although stickleback from Cist did not span the full range of phenotypes from other populations, missing the upper extreme of shape PC2 (Figure 2.4). All 95% confidence ellipses for the three ecotypes and Cist in the body shape analysis were at least partially overlapping (Figure 2.4). Fish from Cist also displayed armour phenotypes spanning all three distinct population ellipses from the training set in the body armour PCA as well as a ‘smudge’ of partially plated

fish, spanning the phenotypic space between pure resident and anadromous and resident and anomalous populations (Figure 2.6).

The mean centroid size for each ecotype differed according to whether fish were from the training set or Cist. Anadromous fish from Cist were significantly smaller than anadromous fish from the training set (Welch's t-test: t = -3.0, df = 13.2, $p < 0.05$), resident fish were also significantly smaller in Cist than in the training set (Welch's t-test: t = -5.70, df = 142.8, $p < 0.001$) and anomalous fish were significantly larger in Cist than in the training set (Welch's t-test: t = 3.63, df = 26, $p < 0.01$), Figure 2.5.

An LDA on all morphological data combined demonstrated that variation in body armour played the largest role in separating ecotypes. Linear discriminant 1 (LD1), which accounted for 97% of between group differences, largely described variation in lateral plate number, with differences in the positioning of facial features and the caudal fin and anal fin also making small contributions (Figure 2.7). Linear discriminant 2 (LD2), accounting for 3% of between group differences, described variation in the lengths of the second dorsal spine, pelvic spine and pelvis (Figure 2.7). LD1 largely separated the anadromous ecotype from the other two, while LD2 separated the resident and anomalous fish.

The LDA identified strong morphological separation between anomalous, resident and anadromous ecotypes in the training set, with the three ecotypes clustering distinctly and separately with no overlap in 95% confidence ellipses (Figure 2.7). Jack-knifed validation indicated that the LDA was highly accurate in classifying stickleback from the training set into the correct group, with 100% of its predictions being correct (n=260). When the discriminant function was

applied to stickleback from Cist, 5.3% of individuals fell inside the anadromous 95% confidence ellipse created by the training set, 29.5% inside the resident ellipse and 20.0% inside the anomalous ellipse, while the remaining 46% fell outside all ellipses, with 15.3% in-between anadromous and resident, 30.0% between resident and anomalous and 0% between anadromous and anomalous ellipses. (Figure 2.7). Even those individuals within the 95% confidence ellipses of the training set tended to be shifted towards the centre of the overall phenotypic space. The cluster analysis indicated that the optimal number of clusters in the training set was three, with a Dunn index score of 1.99, indicating good separation between clusters. The same cluster analysis applied to Cist stickleback also indicated an optimum of three clusters, but with a much lower Dunn index score of 0.29, implying considerably less certainty in the allocation of individuals to clusters.

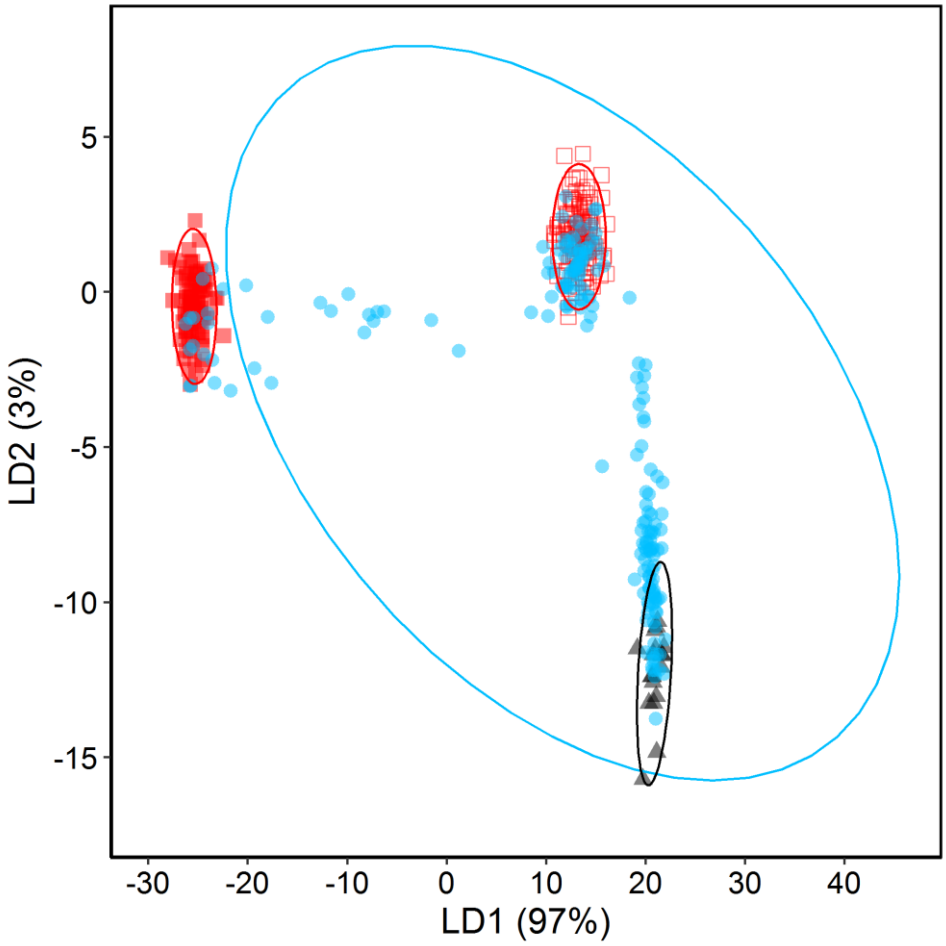

Figure 2.7 LDA on stickleback morphological characteristics. Dispersal of stickleback phenotypes along linear discriminants 1 (LD1) and 2 (LD2) of a linear discriminant analysis (LDA) on all body shape, size and armour variables. The discriminant function was created using a training set including all anomalous stickleback from Scad (grey triangles), all resident stickleback (open red squares) and all anadromous stickleback (closed red squares) from the six species-pair lagoons, but excluding the single partially plated individual from Faik. The grouping variable comprised anomalous, resident and anadromous categories. The discriminant function was then used to predict where stickleback from Cist (blue circles) fell relative to the three pure ecotypes. LD1 is strongly associated with plate number and the positioning of facial features, while LD2 is mostly correlated with the length of the second dorsal spine and size of the pelvis. Ellipses represent 95% confidence ellipses.

2.4.3.2 *Genetic separation in Cist*

Of the Cist stickleback that were genotyped (n=68), 28% possessed a heterozygous *Eda* genotype, compared with just 3% from species-pair locations (Table 2.2). Most of the heterozygous individuals from Cist had a partially-plated phenotype, with a few resident and anadromous individuals also displaying heterozygosity. Anomalous fish from Cist were fixed for the Eda^L allele. A higher proportion of fish with apparently resident and anadromous phenotypes were heterozygous in Cist (16% and 27% respectively) than in the species-pair lagoons (0% and 3% respectively), Table 2.2. As a consequence, the proportion of variance explained by *Eda* was significantly lower in Cist (78%) than in the species-pair lagoons (99%), (Mann-Whitney U test: U = 1986, N_{cist} = 68 N_{pairs} = 64, $p < 0.05$).

2.4.4 Spatial distribution of stickleback ecotypes in Cist

Trapping data from Cist demonstrated that when morphologically hybrid fish are excluded, the proportion of salt (anadromous and resident) to freshwater (anomalous) ecotypes decreased with distance from the sea (binomial GLM: LR_1 = 32.9, $p < 0.0001$, Figure 2.8).

To directly analyse the distribution of ecotypes in Cist; occurrence data were analysed with number of fish caught at each sample site as the response variable and ecotype and distance from the sea as predictor variables. The proportion of the three ecotypes and their morphological hybrids varied significantly with distance from the sea, as identified by a significant interaction between ecotype and distance in the model (negative binomial GLM: LR_1 = 24.651, $p < 0.0001$). Anadromous fish occurred mostly within a few hundred meters from the sea,

resident and resident-anadromous intermediates occurred broadly across most of the transect, but in higher proportions closer to the sea while anomalous and anomalous-resident intermediates occurred in highest proportions furthest from the sea, and were totally absent at the mouth of the loch (Figure 2.8). The ecotype term was also significant in its own right in this model (negative binomial GLM: $LR_4 = 11.19$, $p < 0.05$) reflecting the fact that some ecotypes were more common than others in our sample of stickleback from Cist (out of 190 stickleback, ten were classified by the LDA as anadromous, 29 were resident-anadromous intermediates, 56 were resident, 57 were resident-anomalous intermediates and 38 were anomalous).

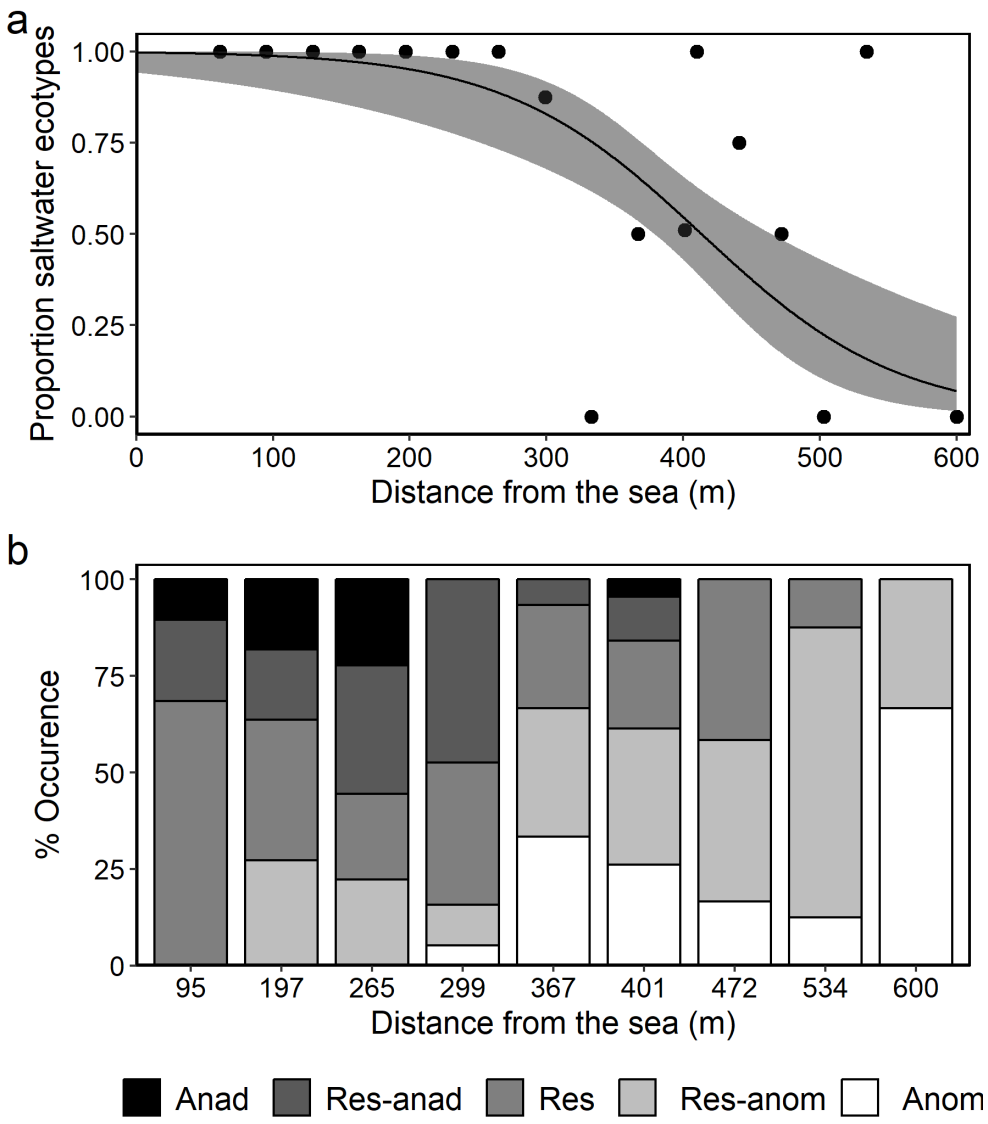

Figure 2.8 Dispersal and spatial segregation of ecotypes in Cist. (a) Proportions of salt : freshwater stickleback in Cist (black circles) and the fit of a binomial GLM with the proportion of salt : freshwater stickleback as the response variable and distance from the sea as the predictor variable. The models associated standard errors are represented by the grey ribbon. (b) Spatial segregation of ecotypes along a 600m transect spanning the tri-ecotype contact zone in Cist. Individuals were classified into ecotypes based on the LDA (Figure 2.7) with individuals falling within training set ellipses belonging to their respective 'pure' ecotype and those falling outside ellipses being considered intermediates with the two parental ecotypes taken as the two closest 'pure' ellipses. Ecotypes are displayed as proportions of the total number of stickleback caught at each site over 5 consecutive days of trapping and for each sample site n = 8-90. Abbreviations represent: 'Anad': anadromous, 'Res-anad': resident-anadromous hybrids, 'Res': resident, 'Res-anom': resident-anomalous hybrids and 'Anom': anomalous.

2.5 Discussion

We find compelling morphological and genetic evidence to suggest that strong RI has developed between resident and anadromous ecotypes in numerous coastal lagoons on North Uist, but that this breaks down in the presence of a third anomalous ecotype. RI between anadromous stickleback and their derived freshwater cousins is well documented in many locations (Higuchi *et al.*, 1996; McPhail, 1994; Rafinski *et al.*, 1989), although it is rare in lacustrine environments (McKinnon *et al.*, 2004; Von Hippel and Weigner, 2004). RI between resident and anadromous stickleback is almost always accompanied by substantial hybridisation (Hendry *et al.*, 2009) and RI as complete as that which appears to exist on North Uist, is extremely rare (Bell *et al.*, 2010). The evolutionary processes that lead to the formation of species-pairs are complex and still not well understood (Richardson *et al.*, 2014; Schluter, 2009), but North Uist stickleback populations provide excellent opportunities to shed light on these.

2.5.1 Evidence for species-pairs

Although we trapped extensively across North Uist, we found that anadromous stickleback do not migrate further than ~1km inland. This was somewhat unexpected given the presence of freshwater adapted stickleback right into the interior of the island, at distances of greater than 10km, through water, from the sea (MacColl *et al.*, 2013; Magalhaes *et al.*, 2016). In addition, there is evidence that anadromous stickleback are capable of oceanic scale migration (Makinen and Merila, 2008; Quinn and Light, 1989). Maybe the additional obstacle of transitioning between physiologically disparate environments limits the possible

migration range (Crossin *et al.*, 2008), although we found no effect of salinity (which requires major physiological changes) on migration. Alternatively, it could be that the optimal conditions for spawning in this species are coastal, or that anadromous fish somehow struggle to compete with freshwater ecotypes. Neither of these explanations make especially good sense, because there are several locations on North Uist where anadromous fish do breed alongside freshwater ecotypes in low salinity brackish, or truly freshwater locations. In any case, interactions between anadromous stickleback and fresh/salt water resident ecotypes, and consequently the occurrence of resident-anadromous species-pairs are spatially restricted to narrow coastal zones <1km from the sea.

Sympatry in coastal regions between resident and anadromous stickleback during the breeding season is common across much of the Holarctic range of the species, and morphological and genetic divergence between populations is almost ubiquitous (McKinnon and Rundle, 2002). Despite this, persistent hybridisation occurs to some extent across nearly all documented contact zones (Higuchi *et al.*, 1996; McPhail, 1994; Rafinski *et al.*, 1989). The occurrence of low-plated resident and completely-plated anadromous morphs in the absence of intermediate partially-plated fish occurs in only two known locations: one in Alaska (Drevecky *et al.*, 2013; Karve *et al.*, 2008) and one in Russia (Ziuganov, 1995). We identified six locations on North Uist in which morphologically intermediate individuals are extremely rare, and are aware of several others. Across the six locations and 242 sampled individuals we found only one phenotypic hybrid. Genetic analysis confirmed that this individual was heterozygous at the *Eda* locus, a gene that was otherwise almost completely fixed for two divergent alleles in resident and anadromous populations. This

suggests that hybrid individuals are readily morphologically detectable by their intermediate plate number, and therefore adult hybrids are probably almost totally absent in the six sampled species-pair lochs on North Uist. It is possible that juvenile hybrids are produced, but eliminated by natural selection prior to reaching adulthood. However, the fact that apparently fully functional adult hybrids are commonplace in Cist, a loch less than 100m from some of the species-pair locations, strongly suggests that this is unlikely to be the case.

There was one other *Eda* heterozygous individual from these six sites with a completely plated and otherwise anadromous phenotype. This could be a second resident-anadromous hybrid, or it could be indicative of longstanding genetic variation at the *Eda* locus within the anadromous population, a well-known phenomenon in many other anadromous and fully marine populations (Bell *et al.*, 2010; Kitano *et al.*, 2008), that is thought to be the reason for the frequency and ease with which marine stickleback colonize freshwater (Barrett and Schluter, 2008; Colosimo *et al.*, 2005; Kingsley and Peichel, 2006; Schluter and Conte, 2009). Either way, it highlights the importance of genetic analyses in studies of divergent morphological characteristics and suggests that although Eda^{L} and Eda^{C} alleles are approaching fixation in some populations on North Uist, hybridisation is probably still occurring at very low levels.

Eda genotype explained a large proportion (85%) of the variation in plate number of North Uist stickleback. This is higher than in most studies (Colosimo *et al.*, 2004; Kitano *et al.*, 2008), but see Lucek *et al.* (2012), and is likely a result of the low proportion of partially plated fish in the genotyped sample (n=15 out of 134 genotyped individuals). The proportion of variance explained by *Eda* was

significantly higher in the species-pair lagoons than in Cist, which again is likely a result of the almost complete fixation of both phenotype and genotype in species-pair populations. Our results do, however, suggest that the complete or almost complete dominance exhibited by the Eda^C allele in some populations (e.g. Cresko *et al*., 2004) is not mirrored in North Uist stickleback. Instead our results indicate a strong dose effect, similar to that found by Colosimo *et al.* (2004), with 67% of *Eda* heterozygotes displaying a partially plated phenotype (Table 2.2).

2.5.2 Evolutionary effects of a third ecotype

Our results highlighted substantially elevated hybridisation in Cist, compared to the six lochs in which the anomalous ecotype is not present. Of all the stickleback caught in Cist, 46% fell outside the training set 95% confidence ellipses in our LDA, indicating that they are morphologically intermediate and presumably hybrids. Even Cist individuals within the ellipses were shifted towards the centre of the phenotypic space, suggesting that they are affected by hybridisation. Ravinet *et al.* (2015) identified a similar contact zone in Western Ireland, but without having any geographically independent training set was unable to detect recent (over the previous two generations) hybridisation between ecotypes and concluded, despite morphological evidence for hybridisation, the three ecotypes were reproductively isolated. In our study the existence of defined ecotypes in multiple species-pair or isolated populations, the substantial morphological evidence, and the introgression of the Eda^L allele into the anadromous population in Cist (27% of anadromous fish in Cist carried the Eda^L allele

compared with 3% of anadromous fish from the species-pair locations, Table 2.2) strongly suggests that Cist contains a hybrid swarm.

Adaptive radiation is characterized by rapid early diversification followed by progressively slower rates of species-turnover (McPeek, 2008). This deceleration is thought to be a consequence of the saturation of available ecological niches (Schluter, 2000). However, in the light of this study, perhaps adaptive radiation puts a brake on itself, as it fills ecosystems with closely related ecotypes that may not be fully reproductively isolated from one another, leaving no particular pair free to evolve RI in the absence of other ecotypes. We suggest that our findings accrue evidence strongly suggesting that adaptive radiation can sow the seeds of its own inhibition.

We propose two potential explanations for the mechanisms by which this may occur. Firstly, additional ecotypes may function as intraspecific competitors, driving diverging morphs back towards the same ecological niche. Trophic divergence in stickleback is associated with the evolution of benthic-limnetic species-pairs (Gow *et al.*, 2008; McPhail, 1992), and is commonly coupled with divergence in body shape to accommodate different feeding strategies (McPhail, 1992). On North Uist, anomalous and anadromous stickleback are similar in shape with both ecotypes having a more slender, elongated profile than deeper-bodied resident fish. Anadromous stickleback spend the majority of their existence in open sea (Crivelli and Britton, 1987) and so are likely to have a predominantly pelagic diet whereas resident fish on North Uist live year round in shallow coastal lagoons and probably utilize a much more benthic food source. Divergent dietary preferences could therefore be involved in speciation between

these ecotypes. The in-wash of anomalous fish in Cist, whose form indicates a limnetic diet (Bentzen and McPhail, 1984), could drive both anadromous and resident populations towards a single benthic fitness optimum, causing the breakdown in RI.

A similar phenomenon took place in Enos Lake, British Columbia, when a formerly isolated benthic-limnetic stickleback species-pair collapsed following the introduction of an interspecific competitor (Taylor *et al.*, 2006). However, the collapse of the Enos Lake species-pair and other similar cases (Bhat *et al.*, 2014) involved continuously on-going ecological interactions over a number of years, whereas the anomalous-anadromous interaction in Cist is temporally restricted to the spring breeding season (MacColl *et al.*, 2013). Whether resource competition over this limited time period would be capable of driving such a profound dietary shift is questionable. Furthermore, the dependency of anadromous fish on dietary resources obtained in freshwater following their spawning migration is unknown and many anadromous species do not eat once they enter freshwater, as fat reserves are built up in the sea prior to migration (Doucett *et al.*, 1999).

Alternatively, additional ecotypes may interfere with reinforcement processes operating within species-pairs, causing their collapse. Reinforcement on secondary contact plays a vital role in many speciation events (Hoskin *et al.*, 2005; Moritz *et al.*, 2009; Wang *et al.*, 1997), probably including those in other stickleback populations (McPhail, 1993). Assortative mating is also well documented in stickleback (McKinnon *et al.*, 2004; Nagel and Schluter, 1998) and therefore the accelerated evolution of discriminative assortative mating

genes and their associated species recognition traits in sympatric stickleback populations has the potential to drive reproductive isolation in North Uist species-pairs.

Assortative mating in stickleback is largely driven by differences in body size (McKinnon *et al.*, 2004; Nagel and Schluter, 1998) and we identified obvious divergence in size between the three ecotypes on North Uist. Furthermore, we found significant differences in body size between Cist fish that fell inside the 95% confidence ellipses of the training set and 'pure' fish from the training set itself. Both of the larger ecotypes (anadromous and resident) were significantly smaller in Cist than in the training set, while anomalous fish were larger in Cist. Two possible scenarios, which are not mutually exclusive, could be responsible for this size convergence. Firstly, it may be driven by environmental factors. Anomalous stickleback are generally found in resource poor oligo- or dystrophic environments (Campbell, 1979), whereas resident and anadromous stickleback inhabit more productive, nutrient rich coastal locations (MacColl *et al.*, 2013; Magalhaes *et al.*, 2016). Resources in Cist may thus be better than average for anomalous fish, but poorer than average for resident and anadromous ecotypes, which could drive convergence in size and consequently a reduction in the efficacy of reinforcement. Alternatively, the reduced separation in size in Cist could be a direct consequence of the introgression of size genes between ecotypes, facilitated by hybridisation (McKinnon and Rundle, 2002; McPhail, 1977). Either way, the reduction in size differences could have contributed to the reduced assortative mating in Cist.

Isolated anomalous stickleback in Scad, the probable source of anomalous ecotypes in Cist, are smaller than resident and anadromous ecotypes, both in Cist and the species-pair lochs. They would, therefore, be unlikely to cause direct confusion in mate selection between resident and anadromous populations because they are not phenotypically intermediate between resident and anadromous forms. Instead, they are more similar in size (and also armour characteristics) to resident fish, making it likely that initial gene flow in Cist was between anomalous and resident fish. Anomalous fish, which are washed into Cist from upstream, probably lack strong mating preferences for their own ecotype because they do not generally encounter other ecotypes. Assuming mate preference is heritable in stickleback (Bakker, 1993), hybridisation between anomalous and resident fish in Cist would result in the introgression of 'indiscriminate' mate choice genes into the resident gene pool as well as the breakdown of diverged assortative mating genes in the resident population via recombination, which could in turn explain why resident and anadromous fish appear to hybridise regularly in Cist, but not in the absence of anomalous fish.

If anomalous-resident hybridisation enabled subsequent resident-anadromous hybridisation, back-crossing would eventually introgress anomalous genes into the anadromous population, theoretically facilitating anomalous-anadromous hybridisation. However, our LDA did not detect stickleback in Cist with a direct anomalous-anadromous phenotype, i.e. individuals scoring intermediately on both LD1 and LD2 (Figure 2.7). Perhaps anomalous-anadromous hybridisation does not occur, but if it does, such hybrids may be rapidly eliminated by selection, or may be difficult to detect in our analyses. LD1 was mostly associated with plate number and LD2 with pelvis size and dorsal spine length.

Thus, in practice the LDA demonstrates that, in Cist, all partially-plated individuals had a complete pelvis and large dorsal spines and all individuals with an intermediately sized pelvis and dorsal spines had a low plate number. This phenomenon is likely the result of tight linkage disequilibrium between regions of the genome containing *Eda* and *Pitx1* (Hohenlohe *et al.*, 2012), which are involved in the development of lateral plates and pelvic structure respectively (Colosimo *et al.*, 2005; Shapiro *et al.*, 2004).

2.5.3 Spatial segregation of ecotypes across a tri-ecotype contact zone

We showed that despite the direct contact between three ecotypes and evidence for extensive hybridisation in Cist, there is strong spatial segregation of ecotypes across the loch. Anadromous ecotypes occurred in the highest proportions at the mouth of the loch, while anomalous fish were most common at the inland extreme. Fine scale population structuring is well documented among stickleback, although it has rarely been investigated in freshwater-saline transition zones (Berner *et al.*, 2009; Snowberg and Bolnick, 2012), but see Vines *et al.* (2016). Small scale spatial isolation of ecotypes is generally associated with RI but we have identified it in Cist despite evidence for substantial hybridisation. Our study therefore demonstrates that spatial segregation alone does not necessarily lead to RI in stickleback and adds to the growing body of evidence which suggests that *in situ* sympatric speciation in this species is likely to be rare, if it occurs at all (Hendry, 2009). These findings, however, also suggest that the contact zone between ecotypes in Cist is particularly narrow (with sympatry between all three ecotypes only occurring across a 300m stretch of our 600m transect). Limited dispersal of ecotypes

during the breeding season could substantially limit the size of stickleback hybrid zones and contribute to fine scale inter-population structure in apparently admixed regions.

2.5.4 Conclusions

The bimodal distribution of lateral plate phenotypes coupled with the fact that determinate Eda^{L} and Eda^{C} alleles are approaching fixation in resident and anadromous populations respectively, and the finding that functional adult hybrids occur in other locations indicates that, across the six species-pair lochs in this study, resident and anadromous populations form reproductively isolated and evolutionarily distinct entities. Opposing findings in Cist suggest that in the presence of the anomalous ecotype this resident-anadromous RI does not form or is reversed. To the best of our knowledge this is the first evidence that the presence of alternative ecotypes within the same species can destabilize speciation. We believe this highlights potentially inhibitory effects of adaptive radiation itself and gives us valuable insight into the processes that drive speciation and maintain RI on secondary contact, as well as highlighting the fragile nature of developing isolation and the volatility of the speciation process.

CHAPTER 3 : ORIGINS OF DIVERGENCE IN STRONGLY ISOLATED STICKLEBACK ECOTYPES ARE PROBABLY ANCIENT AND ALLOPATRIC

3.1 Abstract

The sympatric co-existence of closely related but reproductively isolated 'species-pairs' represents a biological conundrum. Ecological speciation has become a popular model to account for the evolution of such pairs, but genetic data is beginning to suggest that currently sympatric taxa, which might appear to have diverged *in situ*, are often experiencing secondary contact following a long period of allopatry. We use a combination of palaeoecological and genetic data to test two competing hypotheses to explain the existence of strongly reproductively isolated species-pairs of three-spined stickleback (*Gasterosteus aculeatus*) in coastal lagoons on North Uist, Scottish Western Isles. First, we reconstruct historical periods of lagoon-sea connectivity over the Holocene to assess potential for a recent 'double-invasion' intersected by a period of evolution with spatial isolation. Second, we investigate the genetic background of stickleback ecotypes on North Uist and use mitochondrial sequence data to estimate divergence times. We find no evidence for recent parapatric divergence related to changes in relative sea level (RSL), and instead show that divergence in species-pairs is probably related to secondary contact between two largely allopatric, highly diverged ancient mitochondrial lineages.

3.2 Introduction

Ecological speciation has become a very popular model to account for apparent divergence and reproductive isolation (RI) between closely related taxa (Nosil, 2012; Rundle and Nosil, 2005; Schluter, 1996; 2009). However, increasing resolution of genetic data is beginning to suggest that the underlying genetic basis of RI may be much more ancient (Feder *et al.*, 2005), and that currently sympatric taxa, which might appear to have diverged *in situ*, are in fact experiencing secondary contact following a long period of allopatry (Bernatchez and Dodson, 1990; Feder *et al.*, 2003; Foote and Morin, 2015; Kuehne *et al.*, 2007). Whilst ecological speciation is popular in theory, empirical examples often describe partial divergence rather than complete speciation (Hendry, 2009). A classic example of ecological speciation is that of three-spined stickleback (*Gasterosteus aculeatus*), hereafter 'stickleback' (Schluter, 1996; Schluter and McPhail, 1992), but stickleback speciation is seldom complete. Throughout their Holarctic range, pairs of sympatric freshwater or saltwater resident and anadromous stickleback are common, and morphological divergence between ecotypes is ubiquitous (Bell *et al.*, 2004). However, despite sometimes strong RI (Hagen, 1967; Jones *et al.*, 2006; McKinnon and Rundle, 2002), hybridisation typically occurs readily across parapatric contact zones (Hay and McPhail, 2000; Jones *et al.*, 2006). Consequently, it is becoming increasingly apparent that while ecologically derived selection frequently causes divergence, it rarely results in complete speciation (Hendry, 2009; Hendry *et al.*, 2009).

The most unambiguous example of speciation to completion in stickleback is between Pacific Ocean and Japan Sea populations (Higuchi and Goto, 1996) and this is a clear example of intrinsic genetic speciation that is allopatric and ancient in origin (Kitano *et al.*, 2007; Kitano *et al.*, 2009). Strong RI with unusually low hybridisation is also present in benthic-limnetic species-pairs around the Strait of Georgia, BC, Canada (Gow *et al.*, 2008; McPhail, 1992), although it is relatively easily reversed (Taylor *et al.*, 2006). Ecological factors are probably important for benthic-limnetic pairs (McKinnon and Rundle, 2002; Schluter and McPhail, 1992), which were initially thought to have resulted from a 'double-invasion' by a single homogenous marine population, which included a period of spatial isolation facilitated by post-glacial relative sea-level (RSL) fluctuations (McPhail, 1993; Taylor and McPhail, 2000). However, RSL reconstructions for the area are not consistent with a 'double-invasion' (Friele and Hutchinson, 1993; Josenhans *et al.*, 1997) and the probable marine founder population was not homogenous, but rather was experiencing secondary contact between ancient mitochondrial lineages (Johnson and Taylor, 2004; Orti *et al.*, 1994; Taylor and McPhail, 1999), suggesting the underlying genetic basis for benthic-limnetic speciation may well be considerably older (Magalhaes and MacColl, unpublished data). Prior genetic differentiation may, therefore, underlie certain cases of superficially ecological speciation in stickleback.

Assessing the role of spatial isolation in speciation is notoriously problematic because inferring historic population spatial distributions is difficult over long time-scales (Kozak *et al.*, 2008). However, for speciation relating to marine-freshwater invasions such as in many post-glacial fish, estimates of past lake-sea connectivity can be reconstructed using sediment elemental composition

(Chague-Goff *et al.*, 2016; Ziegler *et al.*, 2008) and diatom assemblages (Fritz *et al.*, 1991) from sediment deposited on the lakebed (Talbot and Laerdal, 2000). This allows periods of potential colonisation and spatial isolation to be reconstructed and precisely dated (via radiocarbon dating of the sediment), and is a dramatically underutilised resource in speciation research.

Here we explore possible explanations for the evolution of recently discovered, previously unexplored, reproductively isolated resident-anadromous stickleback species-pairs breeding sympatrically in multiple saltwater lagoons on the Scottish island of North Uist, in the absence of intermediate morphs (Chapter 2). We combine genetic and palaeoecological data to test two competing hypotheses to explain the origin of these species-pairs: (1) the 'classic' stickleback model, involving recent ecological speciation that occurred *in situ* during the Holocene, following a double-invasion. (2) An alternative model in which genetic divergence evolved in allopatry, prior to colonisation, and considerably pre-dates the Holocene period. By consideration of putative mechanisms for these two hypotheses we shed light on the underlying mechanisms of speciation in post-glacial fish.

As a mechanism for the first hypothesis we propose that the species-pairs may have arisen via ecological speciation, as a direct result of divergent adaptation to salt- and freshwater. In general, ecologically based divergent selection between salt and freshwater environments does not appear to have given rise to strongly reproductively isolated ecotypes, which do not hybridise, across the rest of the Holarctic (Hendry, 2009). However, ecological divergent selection coupled with a period of spatial isolation during the evolutionary history of species-pairs could

enable more complete speciation (Rundle and Schluter, 2004). Localised geographical isolation could have been facilitated on North Uist by fluctuations in RSL over the Holocene (Jordan *et al.*, 2010). Some analyses of RSL change in the Hebrides suggest a spike ('high-stand') in RSL immediately after deglaciation, followed by RSL receding, before rising again (Lambeck, 1993a; 1995a; Peltier *et al.*, 2002). Such dynamics could have driven a 'double-invasion', such as the one proposed to explain the benthic-limnetic pairs in BC (Taylor and McPhail, 2000), with a period of spatial isolation (allopatry) in-between. This theory makes three predictions. First, the species-pairs originated from multiple colonisations from a single 'stock' marine population, with no prior genetic or behavioural isolation (Rundle and Schluter, 2004). Second, the species-pairs are post-glacial in age as divergence can only have occurred since the deglaciation of North Uist 12,000 - 16,000 YBP (Ballantyne, 2010), and third, the lagoons in question experienced a double peak in RSL during that time. Alternatively, the species-pairs may be the result of secondary contact between older lineages that arose in allopatry. This makes two clear predictions, that there is a differential genetic origin of ecotypes e.g. as in Bernatchez and Dodson (1990), and this prior genetic divergence significantly pre-dates the last glacial retreat.

To test these hypotheses we reconstructed historic saline influxes in North Uist species-pair lagoons from lake sediment cores using analysis of elemental composition, corroborated by diatom assemblages, to assess the potential for a 'double-invasion'. To quantify genetic divergence in the species-pairs we reconstructed mitochondrial phylogenetic relationships across North Uist stickleback (including five species-pairs populations) using composite

cytochrome *b* (cyt *b*) and mitochondrial control region (CR) sequences that are known to clearly resolve divergence between stickleback mitochondrial lineages present in the north Atlantic (Makinen and Merila, 2008), and used these to estimate divergence-times. We further extended our genetic analysis to include allopatric freshwater populations in attempt to understand the wider genetic structure and colonisation history of North Uist stickleback.

3.3 Methods

3.3.1 Study site

North Uist is a small island in the Scottish Western Isles which was likely colonised by stickleback following deglaciation 12,000-16,000 YBP (Ballantyne, 2010). The island comprises a series of interconnected freshwater lochs and saline coastal lagoons, which cover almost one third of the land surface of the island (Figure 3.1, Table 3.1) making it ideal for studying the marine-freshwater radiation of stickleback.

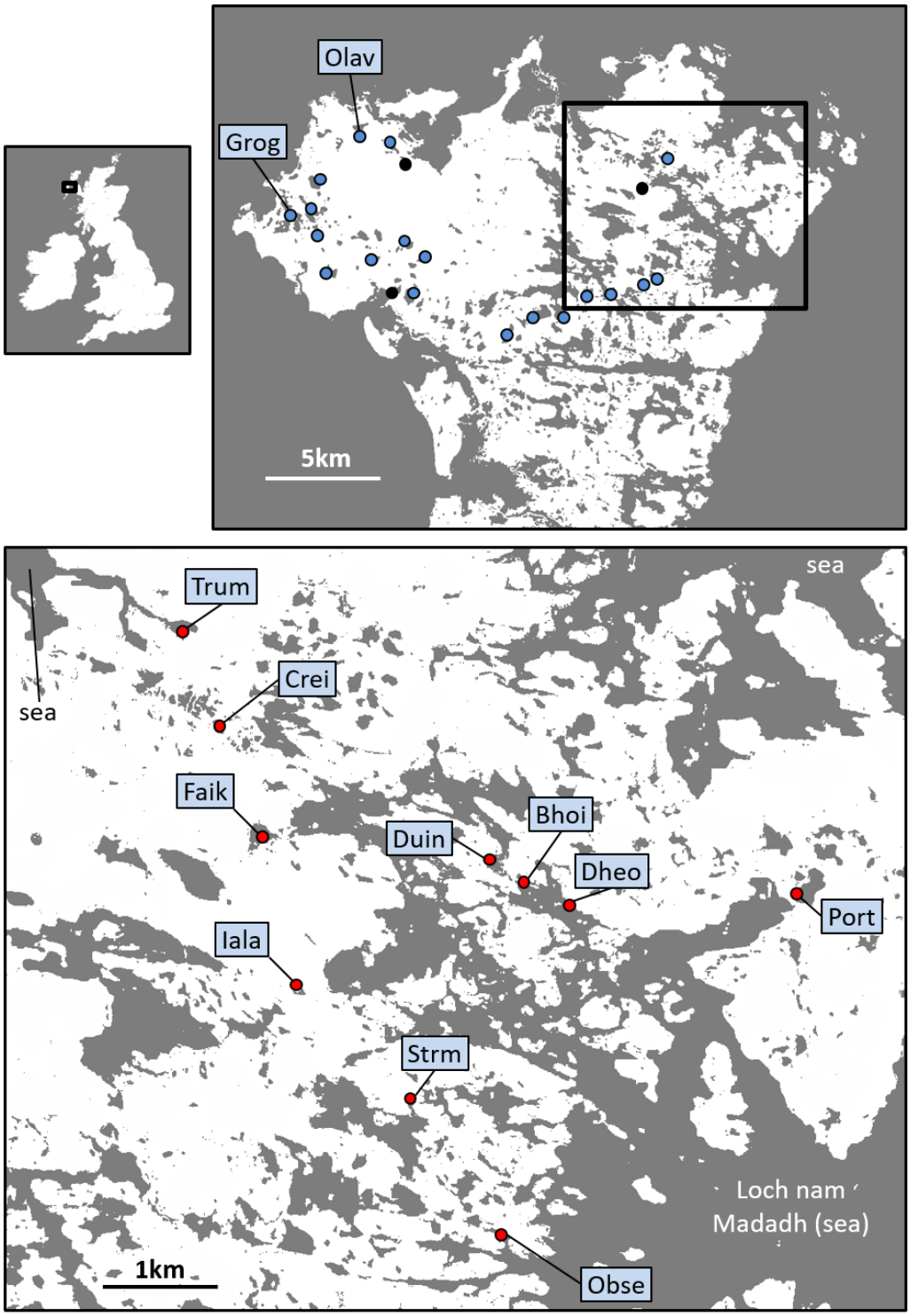

Figure 3.1 Sampling locations Distribution of sampling locations across North Uist. Red circles indicate sites sampled in this work, blue and black circles indicate freshwater and saline locations respectively, sampled by Rahn *et al.* (2016).

Table 3.1 Sample sites. The stickleback populations and sediment coring locations on North Uist used in this study. Sample sizes used for mitochondrial DNA analysis (N_{mt}) are given for resident fish, anadromous fish (curved parentheses) and freshwater fish (square parentheses). Year stickleback (^{s}) and sediment core (^{c}) samples were collected. Stickleback samples collected in 2010 and 2011 were obtained by Rahn *et al.* (2016). Sampling locations are given by latitude followed by longitude.

Loch ID	N_{mt}	Year	Salinity	Location
Obse	10 (10)	2013^{c}, $2015^{c,s}$	brackish	57°36'6"N; 7°10'22"W
Faik[1]	15 (17)	2010^{s}, 2011^{s} $2015^{c,s}$	brackish	57°38'7"N; 7°12'54"W
Strm	5 (2)	$2015^{c,s}$	brackish	57°36'36"N; 7°11'1"W
Iala	N/A	2013^{c}	freshwater	57°37'54"N: 7°12'31"W
Crei	N/A	2013^{c}	freshwater	57°38'41"N; 7°13'33"W
Bhoi	N/A	2015^{c}	marine	57°38'37"N; 7°12'6"W
Dheo	N/A	2015^{c}	marine	57°38'26"N; 7° 9'58"W
Port	N/A	2015^{c}	marine	57°38'1"N; 7° 7'10"W
Duin	10 (3)	2015^{s}	brackish	57°38'35"N; 7°12'40"W
Trum	10 (6)	2015^{s}	brackish	57°39'9"N; 7°14'35"W
Ardh	5 (6)	2011^{s}	freshwater	57°34′48"N; 7°24′48"W
Clac	5 (5)	2011^{s}	freshwater	57°38′14"N; 7°24′45"W
Grog	[5]	2011^{s}	freshwater	57°36′54"N; 7°30′40"W
Eubh	[5]	2011^{s}	freshwater	57°37′6"N; 7°29′42"W
Maga	[5]	2010^{s}	freshwater	57°36′10"N; 7°28′54"W
Host	[5]	2011^{s}	freshwater	57°37′40"N; 7°29′18"W
Sann	[5]	2010^{s}, 2011^{s}	freshwater	57°35′12"N; 7°27′48"W
Olab	[5]	2011^{s}	freshwater	57°39′8"N; 7°26′48"W
Geir	[5]	2011^{s}	freshwater	57°38′34"N; 7°25′18"W
Mgbh	[5]	2010^{s}	freshwater	57°36′6"N; 7°24′36"W
Acha	[5]	2010^{s}	freshwater	57°35′45"N; 7°23′42"W
Moin	[5]	2011^{s}	freshwater	57°35′42"N; 7°25′48"W
Dubh	[5]	2011^{s}	freshwater	57°34′54"N; 7°24′12"W
Torm	[5]	2010^{s}	freshwater	57°33′45"N; 7°19′1"W
Bhar	[5]	2011^{s}	freshwater	57°34′24"N; 7°17′42"W
Mora	[5]	2011^{s}	freshwater	57°34′30"N; 7°16′18"W
Scad	[4]	2011^{s}	freshwater	57°35′6"N; 7°14′10"W
Eile	[5]	2011^{s}	freshwater	57°34′24"N; 7°15′30"W
Daim	[4]	2011^{s}	freshwater	57°35′35"N; 7°12′35"W
Maig	[5]	2011^{s}	freshwater	57°35′42"N; 7°12′6"W
Buai	[5]	2010^{s}	freshwater	57°38′48"N; 7°11′48"W

[1] referred to as "Aileodair" by Rahn *et al.* (2016).

3.3.2 Reconstruction of Holocene relative sea-level change

3.3.2.3 *Lagoon elevation mapping*

Three coastal lagoons, Obse, Faik and Strm (Figure 3.1, Table 3.1), containing resident-anadromous stickleback species-pairs (Chapter 2 and L. L. Dean and A. D. C. MacColl personal observation) were selected for RSL change reconstructions. To identify the lowest point at which sea water could enter each lagoon, the precise elevation (±0.05m) of the underlying rock sills connecting each lagoon to the sea was mapped using detailed digital terrain modelling via Real-Time Kinematic (RTK) Global Navigation Satellite Systems (GNSS) corroborated with Benbecula OS active station GNSS data.

3.3.2.4 *Sediment sample collection and analysis*

To reconstruct Holocene saline influxes, long sediment sequences were extracted from Obse, Faik and Strm. A further five surface sediment sequences of at least 20cm were collected from two freshwater (Iala and Crei) and three saline (Bhoi, Dheo and Port) basins on North Uist (Figure 3.1, Table 3.1), to function as a training set to accurately characterise present freshwater and saline conditions. Sediment sequences were collected using either a 1m Livingston style piston corer or a surface corer suspended from a boat raft. For long cores successively deeper 1m drives with 20cm overlaps were taken until hard basal sediment or bedrock was reached. Cores were retrieved from the deepest part of each basin, located using a handheld echo sounder. Surface cores were extruded and sectioned into 1cm intervals in the field. Long sediment sequences were extruded into half drainpipes and wrapped in cling film in the field, after which they were stored at ~5°C until sectioning approximately one month later.

Following stratigraphic description, long cores were sectioned into 1cm intervals and stored at ~5°C in sealed plastic bags. Samples for energy dispersive X-ray fluorescence (ED-XRF) were air-dried, ground and homogenised prior to analysis. ED-XRF measurements (ppm) of Na, Si, S, Cl, K, Ca, Cu, Zn, Br and Sr, elements with known salinity associations (Chague-Goff *et al.*, 2016; Filikci *et al.*, 2017; Ziegler *et al.*, 2008), were recorded at 5cm intervals for long cores and 2cm intervals for surface cores using a PANalytical Epsilon 3 XLE benchtop ERF energy fluorescence spectrometer. To corroborate elemental-based salinity estimates, samples from Obse were extracted and prepared for diatom analysis following standard procedure (Battarbee *et al.*, 2001). Diatoms (>300 valves per slide) were counted, identified following Krammer and Lange-Bertalot (1988a; b; c) and Snoeijs (1993), and classified according to their salinity tolerances following Van Dam *et al.* (1994).

3.3.2.5 *Radiocarbon dating and depth-age calculations*

Six radiocarbon dates were obtained from areas of interest in the Faik long core. Macrofossil samples were prepared to graphite by the NERC Radiocarbon Facility (East Kilbride) and dated by the SUERC AMS Laboratory. This work was supported by the NERC Radiocarbon Facility NRCF010001 (allocation number: 1942.1015) and publication codes are quoted in Table 3.2. Calibrations were made using CalPal (Weninger *et al.*, 2007).

The sedimentation rate in Faik was calculated by dividing the basal radiocarbon date by the basal depth and its consistency over time was assessed by plotting age-depth correlations for all six radiocarbon dates. Sedimentation rates were likely to be similar across lagoons because of their close proximity and similar

attributes, therefore the timings of fresh-saline transitions in Obse and Strm were estimated by extrapolating the Faik sedimentation rate to these cores.

3.3.3 Stickleback genetic relationships

3.3.3.1 *Stickleback sample collection*

Stickleback were caught from five lagoons (Figure 3.1, Table 3.1) containing resident-anadromous species-pairs using 8 - 30 unbaited minnow traps (Gee traps, Dynamic Aqua, Vancouver) per lagoon, set overnight in water approximately 30-100cm deep. A haphazardly selected sample of up to 20 anadromous and 20 resident stickleback per lagoon were retained and immediately euthanized with an overdose of tricaine methanesulphonate or 'MS222' (400 mg L^{-1}), before being killed by destruction of the brain, in accordance with Schedule One of the UK Home Office regulations. The caudal fin and both pectoral fins were removed from fish immediately and stored in 100% ethanol at -20°C prior to genetic analyses.

3.3.3.2 *DNA extraction, PCR amplification and sequencing*

Genomic DNA from the fin clips of 76 individuals (five to ten resident and two to ten anadromous fish from each lagoon, Table 3.1) was extracted using either Qiagen DNeasy blood and tissue kits, Quanta Biosciences Extracta^{TM} DNA prep for PCR-Tissue kits or following a proteinase K and ethanol precipitation procedure (Goldenberger *et al.*, 1995). DNA was amplified for two mitochondrial regions; cyt *b* and a partial fragment of the D-loop CR. Amplification was carried out using the following primers: cyt *b* forward 5' ATGAAACTTTGGTTCCCTCC 3', cyt *b* reverse 5' CGCTGAGCTACTTTTG

CATGT 3', CR forward 5' CCTTTAGTCCTATAATGCATG 3' and CR reverse 5' CCGTAGCCCATTAGAAAGAA 3' taken from Makinen and Merila (2008). For both regions PCR was carried out in 20µl reaction volumes consisting of 8µl nuclease-free H_20, 9µL 2X Biomix^{TM} red reaction mix (Bioline), 1µl of both forward and reverse primers (10µM) and 1µl of template DNA (approximately 20ng). For cyt *b*, thermocycling was set up as follows: Initial denaturation at 95°C for three minutes, followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for one minute, followed by a final extension at 72°C for five minutes. For CR similar conditions were used except annealing was carried out at 53°C. Amplification success was confirmed by running samples on a 1.5% agarose gel at 110V for ~30 minutes. Cyt *b* and CR fragments were purified using ExoSAP-IT PCR product clean-up kits (Thermo-Fisher Scientific) and sequenced by Source BioScience.

3.3.3.3 *Mitochondrial sequence analysis*

Sequences were aligned using BioEdit version 7.2.5 (Hall, 1999) and electropherograms were inspected and edited for ambiguities by eye. Cyt *b* and CR sequences were then concatenated using Mesquite version 3.04 (Maddison and Maddison, 2015). Composite cyt *b* and CR sequences from this study (1409bp) and from a further 126 North Uist individuals studied by Rahn *et al.* (2016), see Table 3.1 for sampling locations and sample sizes, were subsequently trimmed and re-aligned producing a 1380bp alignment of 202 North Uist individuals. Nucleotide diversity (π), number of haplotypes (h), haplotype diversity (hd), average number of nucleotide differences (k), number of

polymorphic sites, number of parsimony informative sites, and the numbers of fixed differences, shared and total mutations, shared and total mutations in coding regions, shared and total non-synonymous mutations and mean nucleotide differences between pairs of ecotypes / lineages were calculated using the program DnaSP version 5.10.01 (Librado and Rozas, 2009).

The full alignment of 202 concatenated sequences was analysed using a Bayesian phylogenetic approach implemented in MrBayes version 3.2.2 (Ronquist and Huelsenbeck, 2003), with sequence from the Broads S1 *Gasterosteus aculeatus* assembly, downloaded from Ensembl version 84.1 (Yates *et al.*, 2016), accession number: ENSGACG00000020954, as an outgroup. The most appropriate model of nucleotide substitution for the dataset, GTI+I+G, was determined using Akaike information criteria (AIC) (Akaike, 1974) in MrModeltest version 2 (Nylander, 2004), executed in PAUP* version 4.0 (Swofford, 2002), and was implemented in MrBayes. Four independent runs were carried out each with eight MCMC chains 'heated' to a 'temperature' of 0.1, with a relative burnin of 50%. Analyses ran until the average standard deviation of split frequencies fell below 0.01. Traces were visually assessed and marginal likelihoods of the harmonic mean, potential scale reduction factor (PSRF) values and effective sample sizes (ESS) were used to confirm stationarity and parameter convergence between independent runs in MrBayes and Tracer version 1.6 (Rambaut *et al.*, 2014). A 50% majority rule consensus tree was then constructed from the sampled trees.

To further investigate genetic relationships among North Uist stickleback, a median-joining haplotype network was constructed using TCS version 1.21

(Clement *et al.*, 2000) using 95% parsimony criteria, and alternative connections were resolved following standard methods (Crandall and Templeton, 1993). Changing the treatment of gaps from 5th state to missing had no impact on the network analysis.

3.3.3.4 *Divergence-time estimates*

Divergence times were estimated for resident vs. anadromous ectypes and European vs. trans-Atlantic lineages using coalescence-based MCMC simulations on the 76 sequences obtained in this study, implemented in IMa2 (Hey and Nielsen, 2004). Nexus files were converted for IMa2 analysis using PGDSpider version 2.1.0.1 (Lischer and Excoffies, 2012). Four independent runs were carried out from different random number starting seeds. All runs assumed a HKY model of nucleotide substitution with an inheritance scalar of 0.25, as recommended for all mtDNA analyses (Hey and Nielsen, 2004). Forty MCMC chains with a geometric heating scheme of 0.96 /0.9 and a burnin period of 80,000 steps were used in all runs. The upper bounds of parameter prior distributions were initially assumed based on the highest geometric mean of population mutation rates (as specified in the latest IMa2 user guide documentation), calculated using Watterson's estimator in the DnaSP program, and were adjusted after initial runs to ensure the full probability distributions were accounted for, for all parameters. Parameter conversions to demographic units were scaled using the geometric mean of mutation rates for cyt *b* and CR, estimated using the molecular clock calibrations of Makinen and Merila (2008). For our cyt *b* sequence the mutation rate was estimated as (2.045×10^{-8}) x 981bp = 2.01×10^{-5}, and for CR (2.21×10^{-8}) x 428bp = 9.46×10^{-6}, giving a geometric

mean of mutation rates of 1.38 x 10^{-5} /haplotype/year. There is, however, uncertainty surrounding any molecular clock calibrations (Warnock *et al.*, 2012) and therefore parameter conversions to demographic units of time are estimates only.

3.3.4 Statistical analysis

All analyses were carried out using R, version 3.4.1 (R.Core.Team, 2017).

A linear discriminant analysis (LDA), performed using the MASS package, version 7.3-45 (Venables and Ripley, 2002), was used to separate saline and freshwater sediment from the training set of surface cores of known salinity, based on elemental composition. The resulting discriminant function was used to predict historic salinity for long core sediment. All measured elements were scaled and used as predictor variables, with 'freshwater' and 'saline' as grouping variable classes. The accuracy of the LDA in predicting group membership of sediment samples within the training set correctly was determined using a jack-knifed, leave-one-out cross validation approach and the accuracy of long core predictions was determined using maximum posterior probabilities.

Differences between the proportions of mitochondrial lineages across ecotypes was analysed using a chi-squared test with anadromous, resident and freshwater categories for ecotype and trans-Atlantic or European categories for mitochondrial lineage.

3.4 Results

3.4.1 Relative sea-level change reconstructions

The LDA identified clear separation in sediment elemental composition between freshwater and marine environments, with the two groups clustering distinctly and separately along a single linear discriminant axis (LD1, Figure 3.2). Jack-knifed validation indicated that the LDA was 100% accurate in classifying known sediment samples into the correct group (n = 43). The LDA predicted the salinity of all elemental samples from Obse, Faik and Strm long cores with posterior probabilities of > 0.99. LDA predictions indicated that all three currently brackish lagoons transitioned from freshwater conditions during the time spanned by our sediment sequences (Figure 3.2).

Radiocarbon dating indicated that the Faik long core spanned the Holocene period with basal sediment approximately 13,097-13,289 cal. YBP (Table 3.2). The sedimentation rate in Faik was 24.26cm/kyr and almost completely linear (Figure S1, supplementary material). Estimates from extrapolating the sedimentation rate in Faik suggested that basal sediment in Strm was deposited approximately 14,500 cal. YBP and in Obse 6000-7000 cal. YBP. Based on depth-age relationships, the transition to saline conditions in Obse probably occurred within the last 1000 years with no indication of an older saline period. The earliest indication of saline influx in Faik fell between 3,294-3,364 cal. YBP and consistently saline conditions were reached by approximately 2,793-3,244 cal. YBP (Figure 3.2, Table 3.2), again with no indication of a second, older saline period. Strm contained the deepest initial saline section, transitioning from freshwater at a depth of 261cm (Figure 3.2), which corresponded to

approximately 10,750 cal. YBP based on age-depth correlations and sedimentation rates analogous to Faik. The stratified depth and estimated timing of these saline transitions across lagoons is consistent with elevation data, which revealed that Obse was the highest lagoon, followed by Faik, then Strm (1.63m, 1.16m and 0.92m above datum respectively, for detailed topographical maps of the elevations of loch outlets see Figure S2, Figure S3 and Figure S4 of the supplementary material).

Diatom species counts from Obse validated LDA predictions, indicating an identical freshwater - saline transition, with freshwater diatom species throughout the majority of the core and brackish species appearing only in the top 20cm of sediment (Figure 3.2, see Table S1, supplementary material for a full list of identified diatom species).

Table 3.2 Radiocarbon dates. Conventional and calibrated radiocarbon dates for six macrofossil samples from the Faik long core. Depth corresponds to absolute depth from the surface of the lake bed, Rc: radiocarbon.

Lagoon	Drive	Depth (cm)	Conventional Rc age (years BP ± 1σ)	Calibrated Rc age (years BP ± 1σ)	Publication code
Faik	D3	74-75	2793 ± 35	2900 ± 41	SUERC-67962
Faik	D3	92-93	3209 ± 35	3430 ± 30	SUERC-67963
Faik	D3	112-113	3329 ± 35	3560 ± 65	SUERC-67967
Faik	D3	152-153	5815 ± 40	6168 ± 51	UCIAMS-176364
Faik	D5	303-305	10039 ± 41	11562 ± 152	SUERC-67968
Faik	D5	319-320	11294 ± 44	13193 ± 96	SUERC-67969

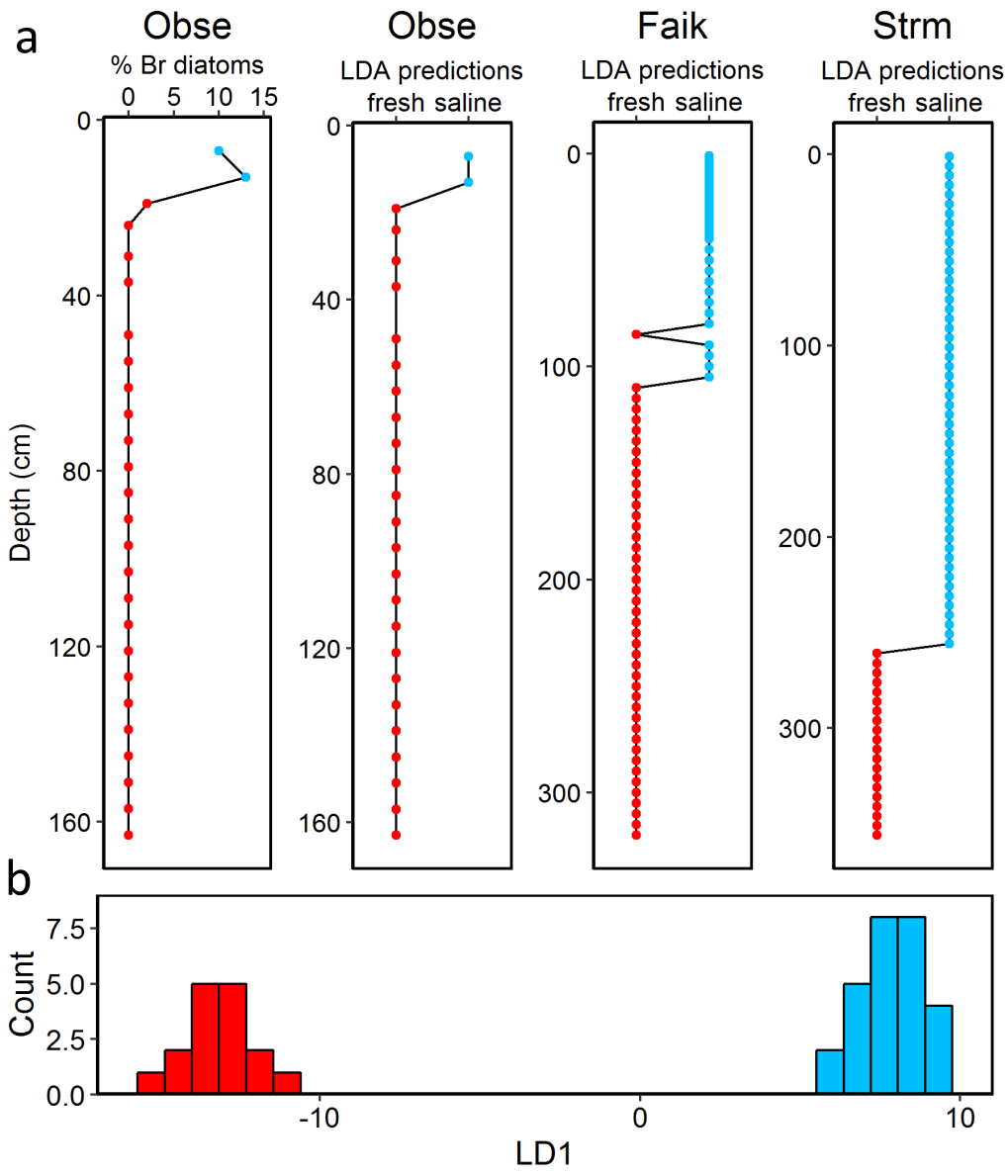

Figure 3.2 Salinity reconstructions for currently brackish North Uist lagoons. (a) Models of past salinity for Obse, Faik and Strm based on the percentage of brackish diatom species (% Br diatoms) and the predictions of a linear discriminant analysis of lake sediment elemental composition (LDA predictions). For the LDA, red circles correspond to 'freshwater' conditions and blue circles to 'saline' conditions. For diatom count blue circles indicate > 5% brackish *Cocconeis* diatom species and red circles < 5%. (b) Separation of fresh (red bars) and marine (blue bars) waterbodies along linear discriminant one (LD1) of a linear discriminant analyses (LDA) based on sediment elemental composition used to classify long core sediment in (a).

3.4.2 Genetic diversity

Seventy six concatenated cyt *b* (981 bp) and CR (428 bp) sequences (total length 1409 bp) from 45 resident and 31 anadromous North Uist stickleback were amplified and sequenced successfully. Cyt *b* and CR sequences were submitted separately to gene bank under accession numbers MG602878-MG602914 and MG602915-MG602951 respectively. These comprised 37 combined (cyt *b* + CR) haplotypes (hd = 0.93 ± 0.02), 19 of which are to the best of our knowledge previously undescribed. A further 126 North Uist mtDNA sequences were obtained from Rahn *et al*. (2016), which resulted in a trimmed sequence length of 1380bp containing 72 polymorphic sites and 34 parsimony informative sites. Across all North Uist sequences there were 79 haplotypes (Table 3.3), fifty of which were represented by a single individual and only 18 of which were shared by more than two individuals.

Table 3.3 Genetic diversity. Indices of genetic diversity for North Uist stickleback populations. Table shows sample sizes (n), nucleotide diversity (π) ± one standard deviation, number of haplotypes (h), haplotype diversity (hd) ± one standard deviation and the average number of nucleotide differences (*k*).

Ecotype / Lineage	**n**	**π ± SD**	**h**	**hd ± SD**	***k***
Resident	60	0.0035 ± 0.0002	30	0.94 ± 0.02	4.84
Anadromous	49	0.0043 ± 0.0003	22	0.91 ± 0.03	5.96
Freshwater	93	0.0035 ± 0.0002	36	0.95 ± 0.01	4.89
Trans-Atlantic lineage	31	0.0015 ± 0.0002	11	0.82 ± 0.06	2.07
European lineage	171	0.0034 ± 0.0001	68	0.94 ± 0.01	4.62
All populations	202	0.0040 ± 0.0001	79	0.95 ± 0.01	5.49

Genetic diversity was almost identical in resident and freshwater populations, whereas in anadromous fish nucleotide diversity was higher, but haplotype diversity was lower (Table 3.3). The trans-Atlantic lineage had particularly low genetic diversity both in terms of haplotype and nucleotide differences in comparison to both the European lineage and each ecotype individually (Table 3.3). Across all pairs of ecotypes, resident and anadromous populations had the most mean nucleotide differences, but also the highest proportion of shared mutations (Table 3.4). However, there was significantly more genetic differentiation between trans-Atlantic and European lineages than between any pair of ecotypes (Table 3.4). Across the entire data set there were 17 non-synonymous mutations in the protein coding cyt *b* region, and very few of these mutations were shared between populations or lineages (Table 3.4).

Table 3.4 Population genetic comparisons. Comparison of genetic differences between North Uist stickleback ecotypes and mitochondrial lineages. Table shows the sample size for each comparison (n), number of fixed differences, number of shared mutations (with the total number of mutations in square parentheses), number of shared mutations in the cyt *b* coding region (with the total number of mutations in coding regions in square parentheses), the number of shared non-synonymous mutations (with the total number of non-synonymous mutations in square parentheses) and the mean number of nucleotide differences between populations or lineages.

Comparison	n	Fixed differences	Shared mutations	Shared mutations in coding regions	Shared non-synonymous mutations	Mean nucleotide differences
freshwater : resident	153	0	14 [66]	7 [47]	1 [16]	5.00
resident : anadromous	109	0	20 [52]	11 [37]	3 [14]	6.34
anadromous : freshwater	142	0	14 [60]	5 [42]	1 [13]	6.25
trans-Atlantic : European	202	2	3 [77]	1 [51]	1 [17]	8.19

3.4.3 Stickleback phylogenetic relationships

Bayesian phylogenetic analysis on all 202 North Uist sequences, alongside comparisons with previously described haplotypes (Makinen and Merila, 2008), revealed that both trans-Atlantic and European stickleback lineages are present on North Uist (Figure 3.3), and the posterior probability for the separation of these lineages was high (1.00). The four independent runs converged to similar likelihoods ([-3343.84] – [-3353.81]). PSRF values (1.000 – 1.001 for all parameters) and ESS values (> 500 for all parameters) indicated good convergence between independent runs and adequate sampling of all parameters. The haplotype network revealed similar relationships to the Bayesian phylogeny with clearly separated trans-Atlantic and European lineages (Figure 3.4).

The majority of North Uist stickleback (85%) fell within the European lineage, with 15% clustering with the trans-Atlantic lineage (Figure 3.3 and Figure 3.4). Lineage associations were highly disproportionate across ecotypes (Chi-squared test: $X = 49.97$, $df = 2$, $p < 0.0001$), with 47% of anadromous fish, but only 6% and 3% of freshwater and resident fish respectively being non-European (Figure 3.5). Five of the six freshwater individuals falling within the trans-Atlantic lineage were from a single, monophyletic population, Olav, and the sixth a single individual from Grog (Figure 3.1, Table 3.1), both described in Rahn *et al*. (2016). A single individual from Duin, and one from Obse made up the 3% of resident trans-Atlantic stickleback (Figure 3.3).

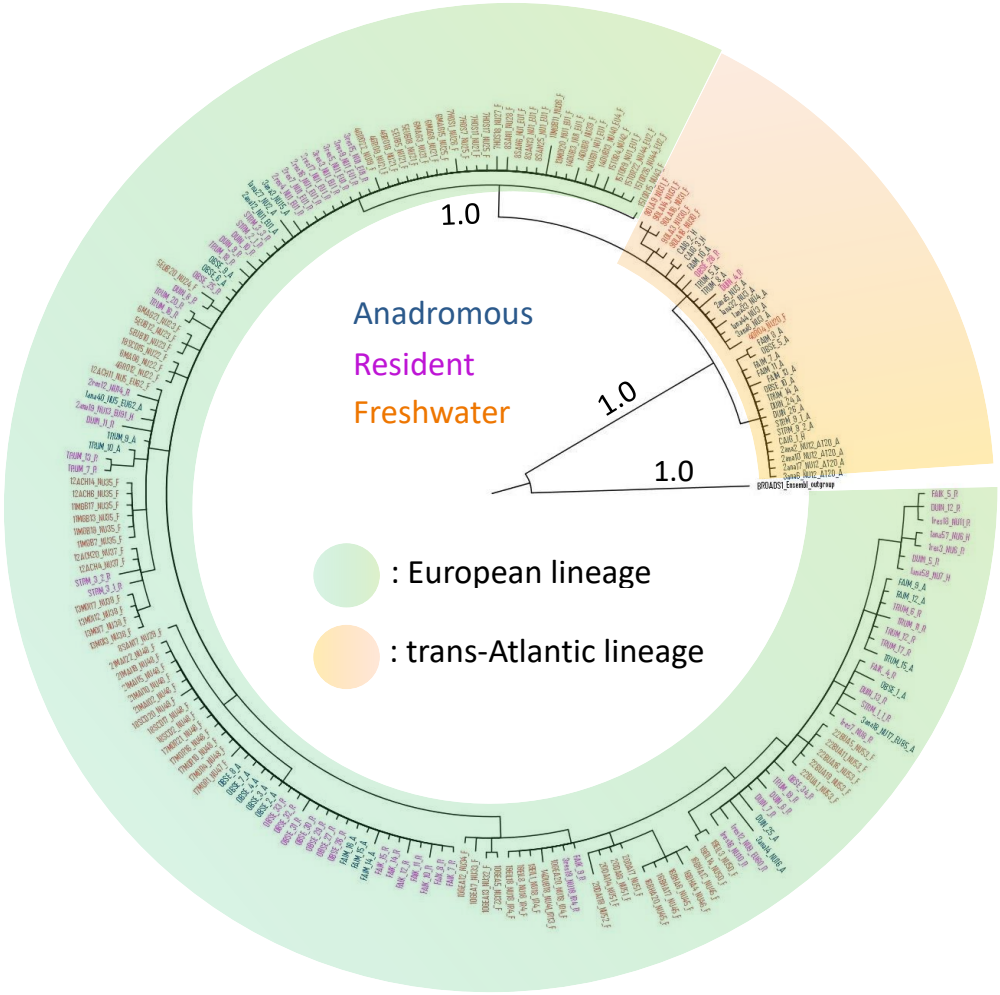

Figure 3.3 Phylogenetic tree of North Uist stickleback. Bayesian 50% majority rule consensus phylogeny of 76 stickleback sequenced in this study and 126 sequenced by Rahn *et al.* (2016), based on concatenated cytochrome *b* (cyt *b*) and mitochondrial control region (CR) sequences. Mitochondrial sequences from the Broads S1 *Gasterosteus aculeatus* assembly (Ensembl) were used for the outgroup. All posterior probabilities greater than 0.98 are given on branches. Anadromous stickleback are shown in red text, resident in purple and freshwater in blue. The European mitochondrial lineage is highlighted in green, and the trans-Atlantic lineage in orange.

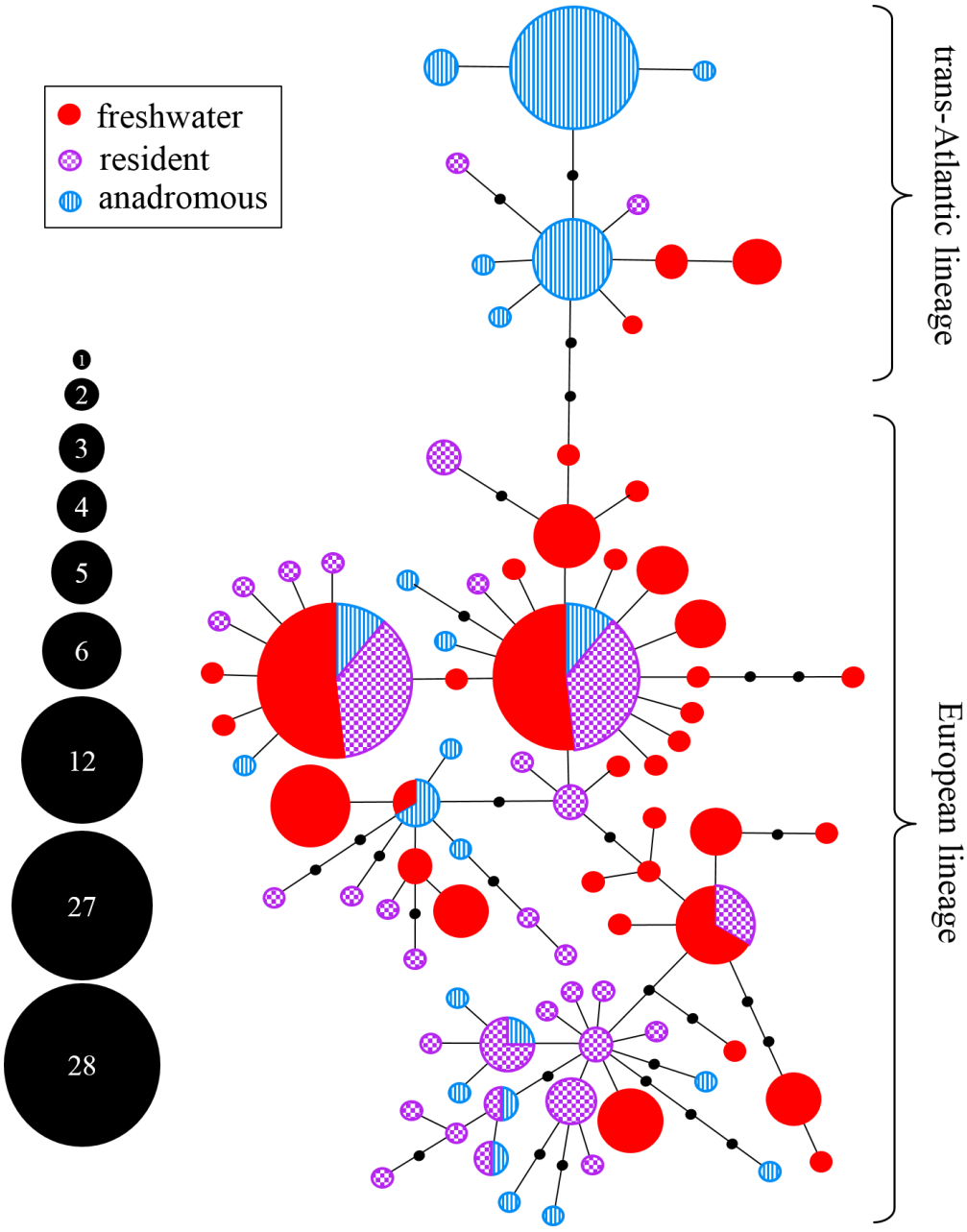

Figure 3.4 Haplotype network. Distribution of North Uist stickleback ecotypes across a haplotype network of composite cyt *b* + CR mitochondrial sequences, based on 95% parsimony criteria. Solid red corresponds to freshwater, purple check to resident, and blue stripes to anadromous stickleback. Black circles represent single mutational steps and haplotype frequencies are indicated by circle sizes.

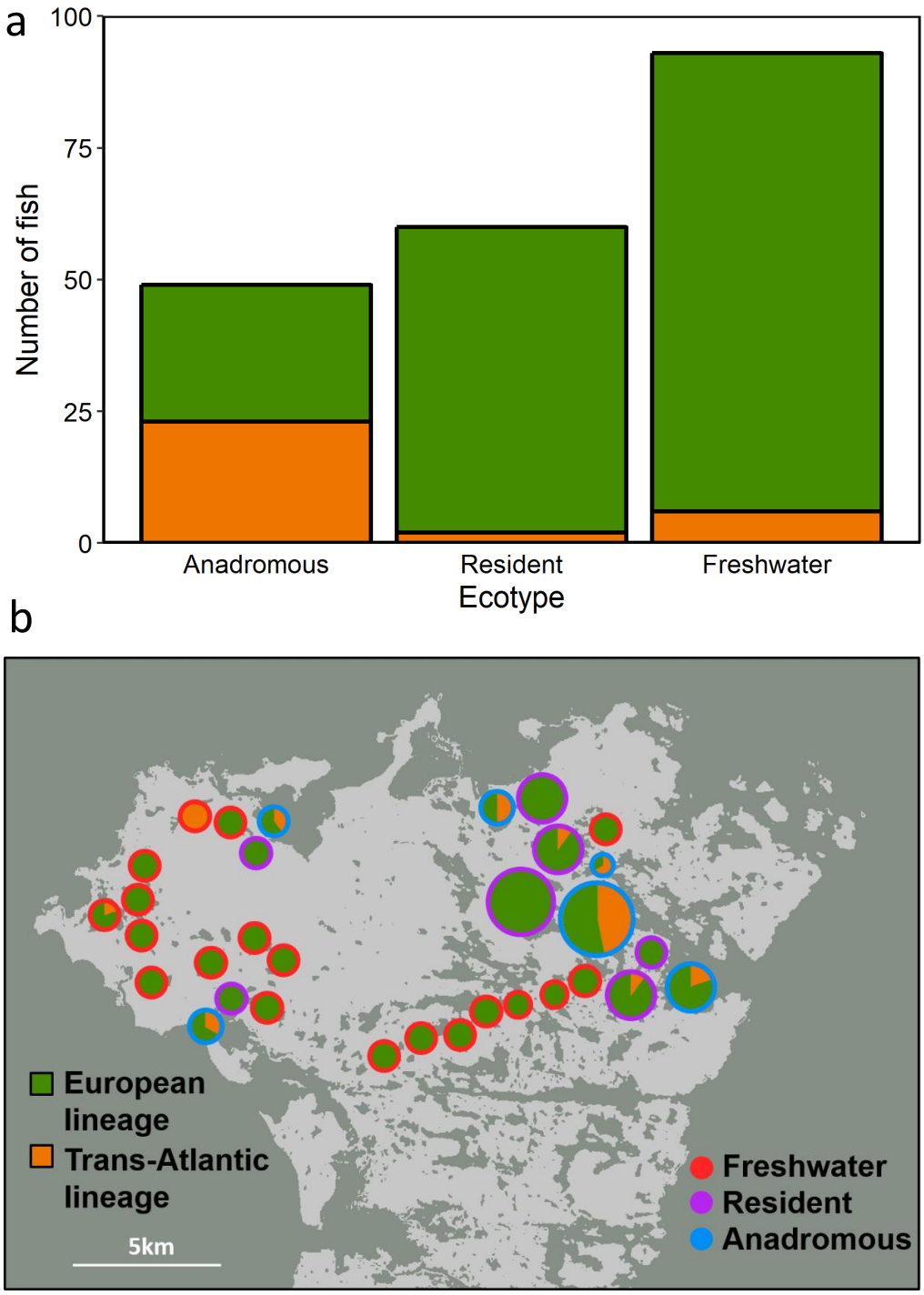

Figure 3.5 Proportions of mitochondrial lineages composing different ecotypes, and their geographical distribution on North Uist. (a) Prevalence of the European (green) and trans-Atlantic (orange) mitochondrial lineages across anadromous, resident and freshwater ecotypes on North Uist. (b) Geographical distribution of ecotypes and lineages across North Uist. Circles indicate sampled stickleback populations: red circles correspond to freshwater, purple to saltwater resident and blue to anadromous populations. Proportions of the European (green) and trans-Atlantic (orange) lineages in each population are indicated by pie charts and chart size reflects sample size.

3.4.4 Divergence time estimates

Posterior probabilities in the IMa2 MCMC coalescence simulations had a unimodal distribution with acceptable 95% high posterior density intervals (HPD) for divergence time (*t*) and time since the most recent common ancestor (TMRCA) parameters (Table 3.5, Figure 3.6). All parameter values converged across four independent runs and therefore average parameter values are shown (Table 3.5). High points and smoothed high points for all posterior probabilities were identical and thus only high points are shown. The divergence time estimates for resident vs anadromous populations were an order of magnitude smaller than those for the European vs trans-Atlantic lineages, with the coalescence simulation suggesting the two ecotypes / lineages on North Uist diverged approximately 12,000 and 143,000 YBP respectively (Table 3.5). Estimates for TMRCA were substantially older than divergence time estimates for both comparisons, but particularly in the resident vs anadromous comparison (Table 3.5).

Table 3.5 Posterior probability of parameter estimates of the IMa2 coalescence simulations and their corresponding parameter conversions. Table shows divergence times (*t*) and time since the most recent common ancestor (TMRCA) and their conversion to years, which were calculated based on a generation time of 1 year and a geometric mean of substitution rates for cyt b and CR combined of 1.38×10^{-5} gene/year. HPD: higher posterior density intervals.

Comparison	***t***	***t* (years)**	**TMRCA**	**TMRCA (years)**
Resident vs anadromous				
High point	0.166	12,056	1.969	142,663
Lower 95% HPD	0.043	3,082	0.906	65,670
Upper 95% HPD	1.808	131,261	3.856	279,438
Trans-Atlantic vs European				
High point	1.643	119,114	2.188	158,515
Lower 95% HPD	0.659	47,773	1.125	81,522
Upper 95% HPD	4.161	301,773	4.150	300,725

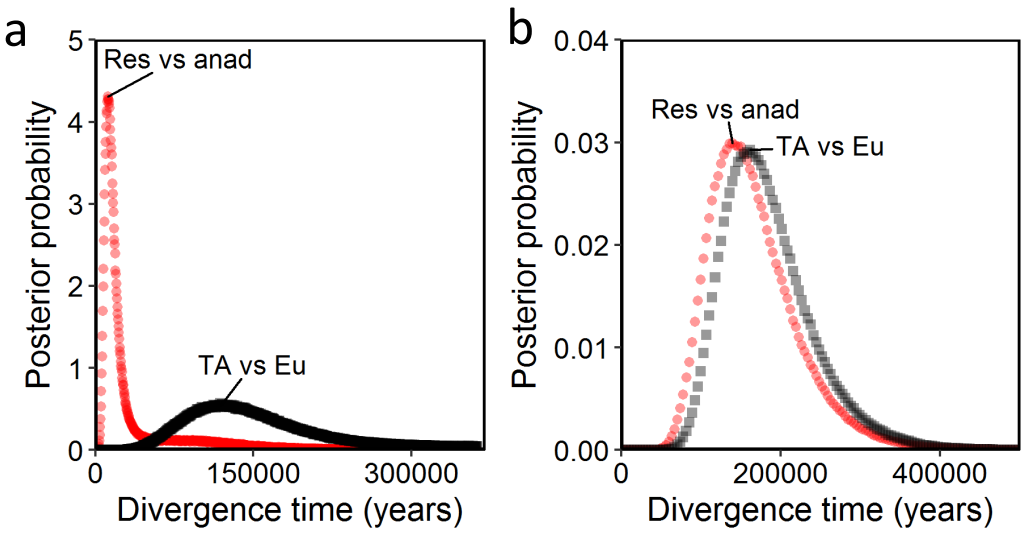

Figure 3.6 Posterior probability distributions of divergence times based on IMa2 coalescence simulations. Graphs show (a) time since divergence (*t*), and (b) the most recent common ancestor (TMRCA) for resident vs. anadromous populations (red circles) and trans-Atlantic vs. European lineages (grey squares) on North Uist. High points for *t* and TMRCA are labelled and 'Res vs anad' refers to the resident – anadromous comparison and 'TA vs Eu' refers to the trans-Atlantic – European lineage comparison in both (a) and (b).

3.5 Discussion

We found no evidence for a double peak in RSL in the species-pair lagoons and no relationship between the timing of saline influxes and genetic divergence, thus our results are not consistent with a 'double-invasion' model for the origin of North Uist species-pairs. Instead, we showed that North Uist is a meeting place for the anciently diverged trans-Atlantic and European mitochondrial lineages, and identified extreme disparity in the proportions of these lineages across ecotypes. Freshwater and resident ecotypes are almost entirely of a single main (European) lineage, while anadromous fish are a mixture of both. Coupled with the apparent strong contemporary RI between anadromous and other fish

(Chapter 2), this pattern is strongly suggestive of allopatric speciation that is comparatively ancient relative to current thinking regarding stickleback (Taylor and McPhail, 2000).

3.5.1 Evidence for a 'double-invasion'

Holocene RSL reconstructions for the Hebrides have indicated a possible double-peak in RSL since the retreat of the Pleistocene glaciers in some locations (Jordan *et al.*, 2010), which may have caused periods of spatial isolation and restricted gene flow in invading anadromous stickleback populations. The hypothesis that RI in North Uist species pairs is a result of a 'double-invasion' caused by these RSL fluctuations resulted in three predictions. First, the species-pairs originated from multiple colonisations from a single 'stock' marine population. However, we showed that the anadromous founder population probably already contained two substantially genetically differentiated lineages, which were experiencing secondary contact. Second, the species-pairs are post-glacial in age. While our divergence-time estimates indicated that there has been post-glacial introgression within species-pairs, genetic differentiation between ecotypes was an order of magnitude older. We also found large inconsistencies in the timings of species-pair divergence and RSL influxes in the three lagoons, which were not consistent with a causal relationship between the two. Resident stickleback diverged from the anadromous population approximately 12,000 YBP, which is considerably earlier than the timing of saltwater influx in Faik (2793 - 3364 cal YBP), and is probably also substantially earlier than the fresh-saline transition in both of the other sediment cores, based on sediment age-depth

correlations. Resident stickleback populations were thus probably established on North Uist prior to any of the lagoons in this study becoming saline.

Third, the 'double-invasion' hypothesis predicted that the lagoons in question experienced a double peak in RSL since the island de-glaciated 12,000 - 16,000 YBP (Ballantyne, 2010). However, our salinity reconstructions showed a single transition from fresh to saline conditions in all three lagoons during that time. We therefore found no evidence for a period of spatial isolation since the island became colonisable for stickleback, although the possibility that a high-stand occurred prior to the time period covered by our cores cannot be discounted. Nonetheless, the apparent lack of spatial isolation, discontinuities between the timing of divergence and RSL changes, and lack of genetic uniformity in the ancestral population makes it extremely unlikely that *in situ* ecologically-based divergent selection alone could have caused the strong RI in North Uist species-pairs (Hendry *et al.*, 2009).

Multiple independent colonisations followed by parallel evolution is thought to be responsible for the global distribution of freshwater and saltwater resident stickleback ecotypes (Colosimo *et al.*, 2004). However, in the most thoroughly studied example of substantial progress to speciation in sticklebacks, the benthic-limnetic pairs in coastal British Columbia, which have previously been thought to have evolved *in situ* (Taylor and McPhail, 2000), there is emerging evidence of an older divergence implying that benthic and limnetic fish across different lakes form monophyletic groups (A. D. C. MacColl, personal communication). Consistent with this idea, stark differences in the predicted timings of fresh – saline transitions across our three lagoons, coupled with the

uniformity of phenotypic and genetic separation within species-pairs (Chapter 2), and the fact that almost all resident stickleback on North Uist are part of a single mitochondrial clade, strongly implies a single origin of the resident ecotype on North Uist, followed by subsequent dispersal. We therefore cannot discount the possibility that an earlier double-invasion occurred in a loch of lower elevation than those investigated here, and this founded extant resident populations.

3.5.2 Evidence for an older, genetic-based split

The mixture of mitochondrial clades in the anadromous population, coupled with a much more recent divergence date for the anadromous-resident, than the European – trans-Atlantic split is consistent with the possibility that a period of introgression followed the secondary contact of these mitochondrial lineages soon after deglaciation. This introgression was strongly asymmetric, with almost no flow of mitochondrial genes into resident or freshwater populations. This is at least partially consistent with four different mechanisms, which are not mutually exclusive: (1) trans-Atlantic stickleback either lack the adaptations necessary to inhabit freshwater or are competitively disadvantaged there, (2) asymmetric mate choice prevents anadromous female x resident male hybridisation, (3) ecologically based selection inhibits backcrossing of any male hybrids to anadromous fish and (4) genetic incompatibilities exist between resident and anadromous ecotypes that make resident/freshwater fish with trans-Atlantic mtDNA inferior.

I consider each of these possibilities in turn: (1) trans-Atlantic stickleback in Europe are probably descended from individuals that crossed the Atlantic. Such

a history might have led to strong selection against freshwater-adaptive mutations, and could mean that trans-Atlantic fish in Europe carry alleles for adaptation to freshwater at much lower frequencies than the European lineage, which, given its current distribution, has probably spent much of its recent evolutionary history in freshwater. A similar scenario in Japanese stickleback lead to only one of two anciently diverged mitochondrial lineages making the transition to freshwater (Ishikawa and Kitano, 2015; Ravinet *et al.*, 2014b). However, trans-Atlantic stickleback have colonised freshwater in a small number of other locations in Europe (Makinen and Merila, 2008), and we identified a very small proportion of freshwater and resident North Uist stickleback of trans-Atlantic origin. These fish were all single individuals from different populations, apart from a single, apparently monophyletic trans-Atlantic freshwater population (Olav). This confirms that the trans-Atlantic lineage is capable of transitioning to freshwater but is consistent with the hypothesis that the trans-Atlantic lineage may generally have been outcompeted by European stickleback on North Uist.

(2) Assortative mating is well documented in stickleback (Milinski and Bakker, 1990; Rundle and Schluter, 1998) and is commonly based on body size (McKinnon *et al.*, 2004), a trait which differs significantly between North Uist ecotypes (Chapter 2), and probably contributes to maintaining RI. In Japanese stickleback species-pairs, mating preferences are asymmetric, with large females only mating with large males, but small females showing no preference (Kitano *et al.*, 2007). It is easy to imagine that the same asymmetry occurs on North Uist, with small resident females being prepared to choose large anadromous males, but large anadromous females avoiding small resident males, especially given

that a larger size is advantageous to males in terms of other preferred traits e.g. larger red throats (Kunzler and Bakker, 2001). This would result in all F1 hybrids having European mtDNA. However, F1 resident-anadromous hybrids are phenotypically more similar to anadromous than resident stickleback (Robertson *et al.*, 2017), making it likely that backcrossing of F1 males to anadromous females would allow trans-Atlantic mtDNA to enter the hybrid population and subsequently introgress into freshwater/resident populations. Thus, it is difficult to account for the scarcity of trans-Atlantic mtDNA in the freshwater and resident populations by mate choice alone. (3) Strong ecologically based selection against anadromous backcrosses could help to prevent introgression of trans-Atlantic mtDNA into the hybrid population. This seems likely given that anadromous backcrosses, like pure anadromous stickleback, would be likely to migrate to sea, where their complement of resident or freshwater genes might inhibit survival in the marine environment (Taylor and Foote, 1991).

(4) Genetic incompatibilities may exist between trans-Atlantic and European lineages causing postzygotic RI. Classic Bateson-Dobzhansky-Muller, or 'BDM' (Bateson, 1909; Dobzhansky, 1937; Muller, 1942), hybrid incompatibilities are often characterised by asymmetric introgression such as that which we identified between stickleback ecotypes (Arntzen *et al.*, 2009; Scascitelli *et al.*, 2010; Welch, 2004). Given the high sequence divergence and ancient divergence time (~119,114 YBP) between trans-Atlantic and European mitochondrial lineages, which were comparable with previous estimates (Makinen and Merila, 2008), we propose mitonuclear conflict as a strong putative candidate for the basis of BDM incompatibilities in North Uist species-pairs. Mitonuclear conflict can be particularly important during speciation,

especially when extended periods of allopatry are involved (Baris *et al.*, 2017; Hill, 2015; Wolff *et al.*, 2014) and trans-Atlantic and European lineages almost certainly evolved largely in allopatry during the Pleistocene, possibly in refugia on opposite sides of the Atlantic (Bigg *et al.*, 2008; Makinen and Merila, 2008). Ecological factors may therefore still have been important as the two lineages could have experienced significantly different environments during the Pleistocene.

If the combination in an individual of European nuclear, but trans-Atlantic mitochondrial sequences were even slightly detrimental, this could produce strong selection for already partially diverged resident/freshwater stickleback to avoid mating with anadromous fish (as this offers a 47% chance of potential mitonuclear conflict in comparison to 3-6% when choosing to mate with fish with a resident or freshwater phenotype). Such conflict could be directly related to European freshwater/resident adapted alleles or it could exist in all anadromous populations where trans-Atlantic and European lineages meet, but be unresolvable due to a lack of phenotypic differentiation between trans-Atlantic anadromous and European anadromous individuals. Trans-Atlantic and European lineages presently meet predominantly in a narrow contact zone along the west coast of the UK and Ireland (Makinen and Merila, 2008; Ravinet *et al.*, 2014a), but, in time, anadromous trans-Atlantic stickleback may become more widespread across Europe, which could have profound consequences for RI in other European marine-freshwater radiations.

3.5.3 Conclusions

Stickleback have long been utilised as a classic example of recent, potentially sympatric, ecological speciation, and localised periods of allopatric divergence resulting from RSL fluctuations have previously been suggested to explain isolated cases of more developed RI (Kitano *et al.*, 2007; Taylor and McPhail, 2000). We show that for North Uist species-pairs a 'double-invasion' with recent divergence in allopatry is unlikely, but rather more developed RI has probably arisen as a result of ancient allopatric divergence and genetic incompatibilities, although these incompatibilities may well stem from ecological adaptations. The large disparity between the proportions of mitochondrial lineages across ecotypes found in this investigation highlights the importance of considering mitonuclear interactions as well as nuclear-nuclear interactions and ecological factors when investigating speciation. Further investigations both into the relationship between divergent ecotypes and mitochondrial lineages on North Uist and across the overlapping range of the trans-Atlantic and European lineages would be likely to greatly aid our understanding of speciation in this model organism.

CHAPTER 4 : REINFORCEMENT OF MATING PREFERENCES DOES NOT DRIVE ASSORTATIVE MATING IN SYMPATRIC STICKLEBACK SPECIES-PAIRS

4.1 Abstract

The progression of speciation following secondary contact and initial gene flow between divergent populations is an important topic in evolutionary biology. If hybridisation is maladaptive, reinforcement may favour the evolution of assortative mating to reduce the production of unfit hybrids. However, the frequency with which reinforcement is important for speciation in nature remains debated. Here we test for signatures of reinforcement and size-assortative mating in highly divergent, strongly reproductively isolated species-pairs of resident and anadromous three-spined stickleback (*Gasterosteus aculeatus*) on the island of North Uist, Scottish Western Isles. We show that stickleback ecotypes mate assortatively, but compared to allopatric populations, we find no evidence that assortative mating is stronger where ecotypes occur sympatrically, and also no evidence that mate choice is related to differences in body size, although there is some suggestion of character displacement in body size. Our results highlight the importance of accounting for other explanations of assortative mating when testing for reinforcement and suggest that speciation by reinforcement may be less common than is often assumed.

4.2 Introduction

Secondary contact and ensuing sympatry between closely related lineages is a particularly interesting topic in the light of evolution because, as well as hybridisation and subsequent homogenisation of populations (Campagna *et al.*, 2014), speciation is, itself, a potential outcome (Brelsford *et al.*, 2011; Noor, 1995). The maintenance and advancement of reproductive isolation (RI) in the face of gene flow (sympatric/parapatric speciation) has been a contentious topic for decades (Berlocher and Feder, 2002; Bird *et al.*, 2012; Bolnick, 2011; Bolnick and Fitzpatrick, 2007; Dieckmann and Doebeli, 1999; Smith *et al.*, 2013; Smith, 1966; Via, 2001), but selection against the maladaptive production of hybrids, i.e. reinforcement (Blair, 1974), provides a potential mechanism for the progression of speciation with gene flow (Rundle and Schluter, 1998). Reinforcement is the adaptive development or exaggeration of assortative mating, that is, the ability to recognize and choose to mate with conspecific individuals, as a result of reduced hybrid fitness (Dobzhansky, 1937; Rice and Hostert, 1993). It is frequently coupled with ecologically derived divergent selection (Kirkpatrick, 2001), which often causes the production of hybrids to be maladaptive (Kopp *et al.*, 2018; Nosil *et al.*, 2003), and is particularly likely when the same trait underlies both divergent selection and assortative mating i.e. so called ‘magic traits’ (Gavrilets, 2004; Servedio *et al.*, 2011; Thibert-Plante and Gavrilets, 2013). Reinforcement can lead to the evolution of exaggerated divergence in characteristics involved in mate recognition and adaptive divergence (reproductive character displacement) (Saetre *et al.*, 1997), including the intensification of assortative mating itself (Doebeli, 2005).

Secondary contact between divergent populations with incomplete postzygotic barriers to RI is common in nature (Campagna *et al.*, 2014; Moritz *et al.*, 2009; Ravinet *et al.*, 2013; Sa-Pinto *et al.*, 2010), indicating that speciation by reinforcement may also be common. However, despite considerable progress in our understanding of this process in recent years (Bird *et al.*, 2012; Kopp *et al.*, 2018), the frequency with which it actually occurs, and the biological factors that promote it remain debated (Servedio, 2004). This is, in part, because assortative mating / RI can develop as a by-product of adaptation to different environments, without the need for hybridisation to be maladaptive (Rice and Hostert, 1993; Vines and Schluter, 2006), making it very difficult to demonstrate conclusively that reinforcement is, or has been occurring. Comparing sympatric and allopatric populations that are otherwise similar can help to disentangle by-product RI from that which has arisen via direct selection because selection to reduce hybridisation can only exist where hybrid production is possible. If reinforcement is occurring, sympatric populations should, therefore, exhibit stronger assortative mating than ecologically and genetically similar allopatric populations. Such comparisons are not always possible (Pfennig and Pfennig, 2009), but where they are they can shed light on the relative frequency and importance of reinforcement for the progression of speciation with gene flow.

Contact between phenotypically and ecologically divergent ecotypes with varying degrees of RI occurs throughout the Holarctic range of the three-spined stickleback (*Gasterosteus aculeatus*, hereafter 'stickleback'), largely as a result of their marine to freshwater radiation (Bell *et al.*, 2004; Jones *et al.*, 2012a; Magalhaes *et al.*, 2016; Taylor and McPhail, 2000). They are an excellent organism for investigating the role of reinforcement in advancing speciation as

they frequently satisfy the conditions necessary for reinforcement to occur, with both ecological divergent selection and assortative mating, based on numerous potentially adaptive traits, being well described (Gow *et al.*, 2007; Ingram *et al.*, 2015; Kozak *et al.*, 2011; Schluter, 1993). Body size is often a particularly important cue for mate recognition (Head *et al.*, 2013; Ishikawa and Mori, 2000; McKinnon *et al.*, 2004; Nagel and Schluter, 1998), and is almost certainly also adaptive (Schluter, 1993), making it a potential 'magic trait' linking size assortative mating and adaptive divergence in stickleback (Conte and Schluter, 2013; MacColl, 2009). Furthermore, genetic coupling linking body size with body size preferences is evident in some stickleback populations (Bay *et al.*, 2017). Character displacement in body size is, thus, likely to be evident in sympatric populations that exhibit size-assortative mating (Schluter and McPhail, 1992). As well as being driven by direct mating preferences, assortative mating in stickleback has also been linked with spatial and temporal segregation of phenotypes (Borzee *et al.*, 2016; Hagen, 1967; Pegoraro *et al.*, 2016; Snowberg and Bolnick, 2012), and may, in practice, be influenced by a combination of direct and indirect factors (Snowberg and Bolnick, 2012).

Hybridisation in stickleback contact zones is often extensive (Hendry *et al.*, 2009), but there are isolated cases where the local geography has probably caused a period of allopatric evolution followed by secondary contact, and RI is often more pronounced in such cases (Higuchi and Goto, 1996; Kitano *et al.*, 2007; McPhail, 1993). Reinforcement is probably important in maintaining these elevated levels of RI, both in benthic-limnetic (Gow *et al.*, 2006; Rundle and Schluter, 1998), but see Albert and Schluter (2004), and Japan Sea anadromous – Pacific Ocean anadromous (Kitano *et al.*, 2007) species-pairs. It is also

implicated in certain freshwater-anadromous contact zones (Hay and McPhail, 1975; Ishikawa and Mori, 2000), although its relative importance across most of the rest of the stickleback radiation is unknown.

Recently, we identified a handful of sympatrically breeding resident-anadromous stickleback species-pairs in coastal lagoons surrounding the island of North Uist in the Scottish Western Isles (Chapter 2). These ecotypes differ markedly in many phenotypic traits, particularly body size (Chapter 2) and preliminary investigation indicates strong reproductive isolation (Chapter 2), which may be a result of secondary contact (Chapter 3). However, the mechanisms maintaining this isolation are currently unexplored. Here we use body size data and no-choice mating trials, focusing on female mating preferences, to test the following hypotheses: (i) species-pairs will exhibit strong assortative mating, but allopatric populations will be less choosy, (ii) mate choice will be related to body size differences and (iii) this will lead to consistent character displacement in body size across species-pairs.

4.3 Methods

4.3.1 Fish collection and husbandry

To assess the occurrence of character displacement in body size across lochs, we measured resident and anadromous fish from six lochs containing species-pairs (Table 4.1). In April-May 2016 and 2017 stickleback were caught using un-baited minnow traps (Gee traps, Dynamic Aqua, Vancouver) set overnight in water 30-100cm deep. Captured stickleback were haphazardly selected and measured for standard length (from the tip of the snout to the tip of the caudal peduncle) using handheld callipers, before being returned to their loch unless

required for other experiments. For use in mate choice trials, fish in breeding condition (males displaying full nuptial colouration and heavily gravid females) were selected from three lochs (Table 4.1): two containing sympatric species-pairs and a third containing an isolated, allopatric freshwater population (details below). These were transported to a rental property on the island, in aerated loch water. Fish were then transferred to bare loch specific stock tanks containing either freshwater (dechlorinated tap water) or ~20-30ppt saltwater, depending on the salinity of source lochs (Table 4.1). Saltwater was either pumped directly from the sea or prepared using Seamix artificial sea water mix and dechlorinated tap water. All fish were fed on washed, defrosted bloodworm once a day and were kept in stock tanks until required for mate choice trials, after which they were anaesthetized using an overdose of MS222 and killed by destruction of the brain, in accordance with Schedule One of UK Home Office regulations.

Table 4.1 Stickleback sample sites. The stickleback populations on North Uist used in this study. Sample sizes are given for resident fish, anadromous fish (curved parentheses) and freshwater fish (square parentheses) for both body size comparisons (N_{bs}) and mate choice trials (N_{mc}). Sampling locations are given in latitude, followed by longitude.

Loch ID	N_{bs}	N_{mc}	Salinity	Location
Dheo	20 (14)	N/A	brackish	57°38'26"N; 7° 9'58"W
Duin	90 (69)	N/A	brackish	57°38'35"N; 7°12'40"W
Trum	30 (20)	N/A	brackish	57°39'9"N; 7°14'35"W
Leod	14 (111)	N/A	brackish	57°32'39"N; 7°20'8"W
Obse	265 (325)	30 (50) [0]	brackish	57°36'6"N; 7°10'22"W
Faik	342 (259)	34 (35) [0]	brackish	57°38'7"N; 7°12'54"W
Reiv	N/A	0 (0) [33]	freshwater	57°36'39"N; 7°30'50"W

4.3.2 Mate choice trials

To assess the presence and strength of assortative mating and reinforcement in species-pairs, female no-choice mating trials were conducted between ecotypes from two lochs containing sympatric resident-anadromous species-pairs (Obse and Faik, Table 4.1). Ideally, comparison in mating preferences between sympatric ecotypes and morphologically and ecologically similar, but allopatric ecotypes would be made. However, there are no known locations on North Uist in which anadromous or resident ecotypes spawn allopatrically and therefore an allopatrically breeding freshwater population (Reiv, Table 4.1), that was morphologically as intermediate as possible between resident and anadromous ecotypes, was selected for mate choice comparisons. All possible comparisons between different ecotypes from different populations were tested in this experiment.

No-choice, as opposed to choice, trials were used as anadromous male stickleback may destroy the nests of smaller resident males in a confined environment, making choice trials impractical. Female no-choice trials generally work well in stickleback, providing a more conservative estimate of mating preferences (Coyne, 1993; Dougherty and Shuker, 2015; Furin *et al.*, 2012). We primarily focus on female choice because this is generally more important than male mate choice for pre-mating isolation is sympatric stickleback species-pairs (Kozak *et al.*, 2009).

Trials were conducted in 55L clear plastic boxes filled with fresh (for freshwater males) or saltwater (for resident and anadromous males) prepared as in stock tanks. Each box contained at least one rock for cover, some aquatic plant material

collected from stickleback source lagoons, 200 seven centimetre long black cotton threads, which could be used as nesting material (Smith and Wootton, 1999), and two large petri-dishes, one filled with sand, and one with gravel collected from nearby lochs, for nesting substrate. Saltwater males were also given an additional large petri dish containing mud, and a seaweed covered rock, both collected from nearby marine environments to encourage them to construct nests. Nesting substrates collected from saline environments were not included in freshwater boxes so as not to alter the salinity of the water, and because these substrates are not generally available in freshwater. After acclimatization for at least 2 hours in stock tanks, males were transferred to individual nesting boxes. Boxes were checked daily for signs of nest construction and a nest was deemed complete when both an entry and exit hole were visible. The substrate on which males chose to build their nests, along with their ecotype, was recorded for each nest in order to investigate potential microhabitat differences in nest location between ecotypes. Males that failed to construct a nest within seven days were replaced.

Following nest completion a single heavily gravid female was introduced to each box in a small plastic jar, which subsequently acted as a refuge for the female during the trial (male courtship in sticklebacks can be aggressive, particularly when a larger male and a smaller female are involved). The behaviour of both stickleback was recorded using a DBPOWER wide-angle waterproof digital video-camera positioned at the opposite end of the box to the nest. Trials began upon first interaction between the male and female (which usually took place within 10 minutes of the female being introduced), and lasted for approximately

40 minutes. If mating had not taken place after this time there is an extremely low likelihood of it ever occurring (Nagel and Schluter, 1998).

After trials were complete females were removed from the boxes, anaesthetized and killed according to Schedule One procedure. If spawning did not occur during the trial, females were stripped of their eggs to confirm readiness to spawn (eggs are easily removed from fully gravid females when gentle pressure is applied to the upper abdomen). Trials in which females could not easily be stripped of their eggs were discarded (this happened only seven times over 107 total trials). Females were blotted, weighed and measured for standard length. In trials where eggs had been laid the nest was removed from the male's box and eggs were carefully removed. Nests were subsequently returned to males, who were given 24 hours to rebuild their nests before they were offered to a subsequent female. Each male was used in a maximum of three trials, separated by at least 24 hours. The order of trials was largely determined by the availability of females, however a male was never offered to a female from the same population twice. Once males had been used in up to three trials they were also anaesthetized and killed according to Schedule One, and measurements of standard length and weight were taken.

Videos were visually analysed using windows media player and the occurrence of spawning (females entering the nest and laying their eggs) was recorded for all trials. Trials in which the male and female failed to interact during the entirety of filming were discarded.

4.3.3 Statistical analysis

All statistical analyses were carried out in R version 3.1.4 (R.Core.Team, 2017). Where linear regression models were used, all numeric variables were centred and scaled prior to analysis and model simplification was conducted using a stepwise top down approach, with the least significant terms removed first. The significance of terms in the model was assessed using likelihood ratio tests or F tests, as appropriate. The goodness-of-fit of the best fitting model was then evaluated using residual and Quantile-Quantile (Q-Q) plots, and models were transformed and re-fitted if the necessary family criteria were violated.

Differences in the preferred substrate for nest construction between male ecotypes were assessed using a chi-squared test. To identify factors affecting spawning probability a generalized linear mixed model (GLMM) with a binomial error structure and logit link function was implemented using the lme4 package, version 1.1-13 (Bates *et al.*, 2015) in R. The occurrence of spawning during the trials was used as a binary response variable, with year (2016 or 2017), absolute difference in body size (mm), female ecotype (freshwater, resident or anadromous), whether both male and female were of the same ecotype (0 or 1), and the interaction between the latter two as fixed effects in the model. To control for the effects of individual males being used in multiple trials the individual male used in each trial was included as a random effect.

Our experiments were designed using anadromous and resident fish from two separate locations (lochs Obse and Faik), to test whether assortative mating between ecotypes would be maintained across populations. Therefore, we repeated the GLMM analysis on the occurrence of spawning using a reduced

data set, which excluded all trials involving males or females from the freshwater loch, Reiv, in order to test for the effects of loch in species-pair trials. The model was specified as above, except the fixed effects were: female ecotype (resident or anadromous), whether or not females and males were of the same ecotype (0 or 1) and whether or not females and males were from the same loch (0 or 1), and the interaction between female ecotype and loch.

To compare the body size of fish used in mate choice trials a linear regression model (LM), with body length as the response variable and ecotype and sex, and the interaction between the two as linear predictors, was used. To investigate the relationship between the body size of resident and anadromous ecotypes across multiple species-pair lochs we used a LM with standard length as the response variable and loch and ecotype, and the interaction between the two as predictor variables. To test for a correlation between the body size of resident and anadromous fish across lochs a t-test based on Pearson's correlation was used.

4.4 Results

4.4.1 Mate choice experiments

A total of 91 successful mating trials (in which males and females interacted during the trial, and females either layed or were easily stripped of their eggs) were conducted (Table 4.2). Overall, spawning occurred in 24 of these 91 trials (26%). Spawning occurred in at least one trial for every possible combination of ecotypes apart from resident males with anadromous or freshwater females.

Table 4.2 Mate choice trial combinations. The number of trials completed for each trial type.

	Female ecotype		
Male ecotype	Anadromous	Resident	Freshwater
Anadromous	22	21	8
Resident	7	13	2
Freshwater	5	8	5

4.4.1.1 *Differences in nest locations*

Males of different ecotypes preferred to build their nests on different substrates ($X^2 = 58.72$, $df = 10$, $p < 0.0001$). Anadromous and resident males were offered five substrates and resident males showed an overwhelming preference for nesting on weed (82%), while anadromous males preferred other substrates in 90% of cases, particularly sand (46%), followed by gravel (20%). Freshwater males were offered three nesting substrates and also chose to nest on sand most frequently (67%), followed by gravel (33%), Figure 4.1.

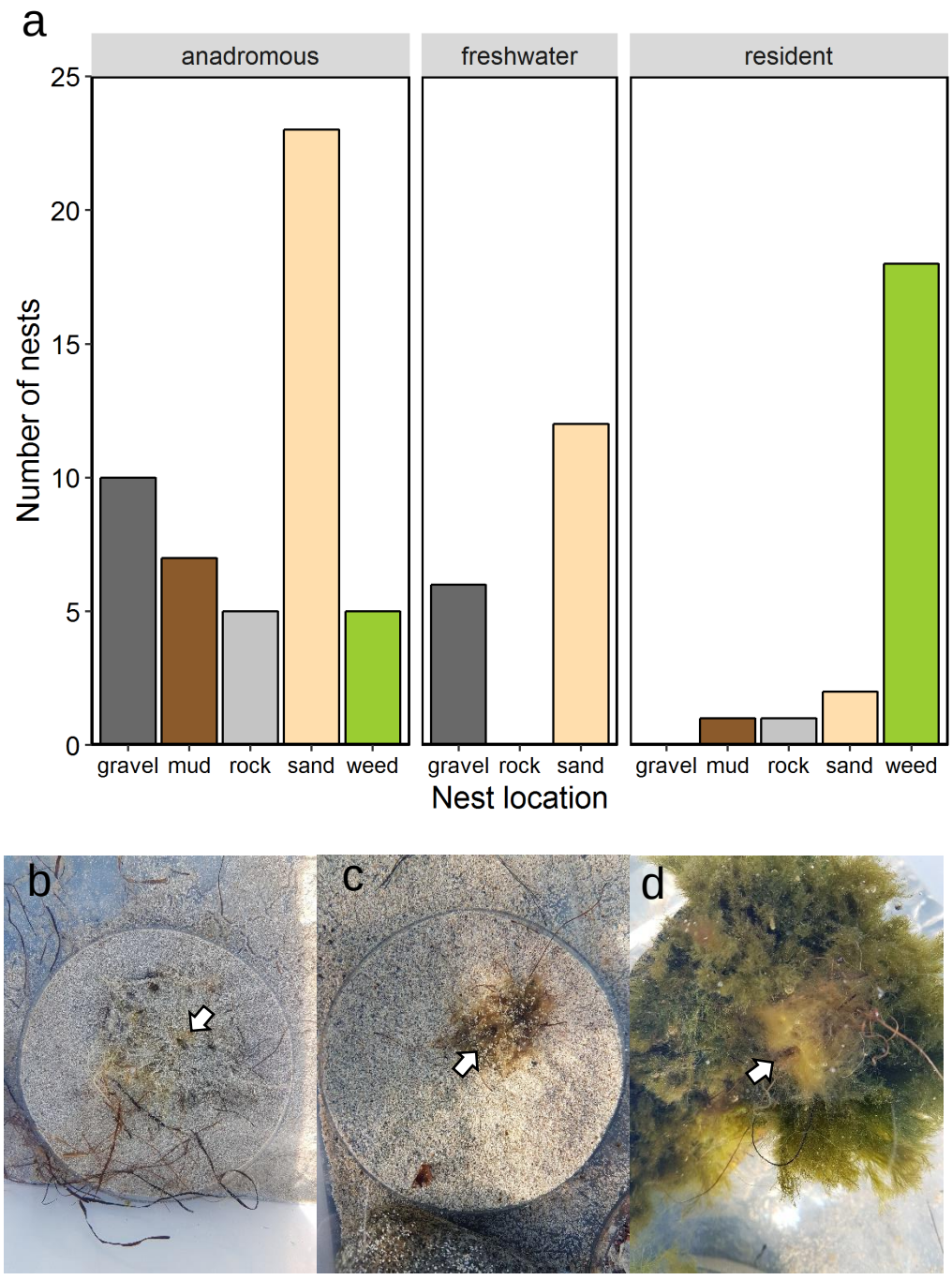

Figure 4.1 Nest locations. (a) Differences in the substrate on which males of different ecotypes chose to build their nests. Examples of nests built on (b) mud, (c) sand and (d) weed during mate-choice trials. Arrows indicate nest entrances.

4.4.1.2 *Assortative mating*

Our results indicated that the three stickleback ecotypes exhibit positive assortative mating as spawning probability was higher with conspecific over heterospecific males (binomial GLMM: $LR_1 = 6.30$, $p = 0.012$, Figure 4.2). This pattern remained consistent in the model that only included trials within species-pairs, with females still being more likely to spawn with conspecific over heterospecific males (binomial GLMM: $LR_1 = 20.02$, $p < 0.0001$). In the species-pairs, the female preference for conspecific males occurred irrespective of whether they were from the same loch as the males (binomial GLMM female ecotype x loch: $LR_1 = 0.03$, $p = 0.87$) and this effect was consistent across both resident and anadromous females (binomial GLMM: $LR_1 = 0.01$, $p = 0.91$).

The probability of spawning was considerably higher in 2016 than 2017 (binomial GLMM: $LR_1 = 16.12$, $p < 0.0001$), and this effect was still present, although slightly weaker, in the species-pairs alone (GLMM: $LR_1 = 7.29$, $p = 0.007$).

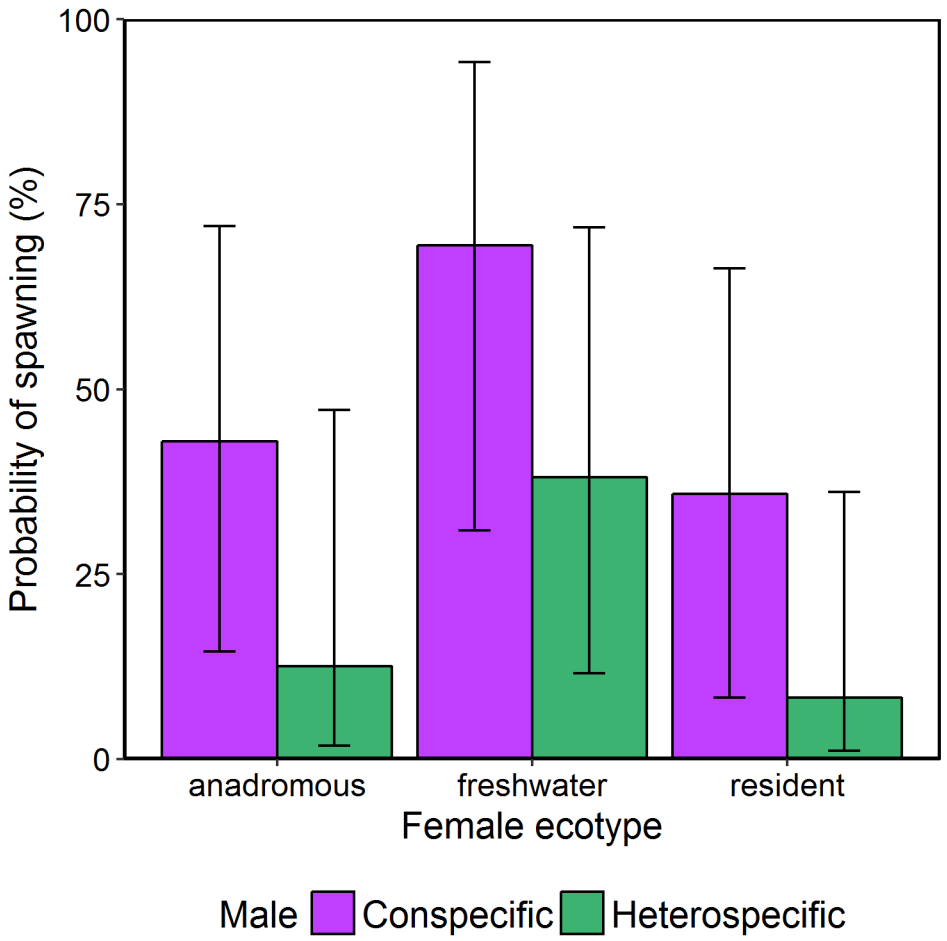

Figure 4.2 Differences in spawning probability across ecotypes. Predicted probabilities of females of different ecotypes spawning with conspecific vs. heterospecific males. Error bars show associated bootstrapped 95% confidence intervals. Predictions are based on a binomial generalized linear mixed model with individual male as a random effect and year, female ecotype and conspecific male as fixed effects. The estimated variance component ± S.D. for the random effect of individual males was 1.27 ± 1.13 in the full model and 553.7 ± 23.53 in the species-pair only model.

4.4.1.3 *Reinforcement*

The strength of assortative mating did not differ between the three ecotypes (binomial GLMM, female ecotype x ecotype same: $LR_2 = 1.55$, $p = 0.46$, Figure 4.2). There were, however, differences between ecotypes in the overall likelihood of females spawning, regardless of the ecotype of the male (binomial GLMM: $LR_2 = 7.03$, $p = 0.029$, Figure 4.2). Re-running the model with resident and anadromous females collapsed into a single level of the female ecotype factor ('saltwater females') confirmed that these two ecotypes did not differ from each other in their overall spawning probability ($X^2 = 0.33$, $df = 1$, $p = 0.56$), and therefore the effect of female ecotype was caused by a greater probability of spawning in the allopatric freshwater ecotype, compared to the sympatric resident and anadromous ecotypes (Figure 4.2).

4.4.1.4 *Size assortative mating*

The body length of the fish used in mate choice experiments differed significantly across the three ecotypes (LM: $F_{2,\ 177} = 421.83$, $p < 0.0001$). Anadromous fish were the largest, residents the smallest and the freshwater ecotype fell centrally between the two (Figure 4.3). Females were larger than males (LM: $F_{1,\ 177} = 88.34$, $p < 0.0001$), but this difference varied heteroscedastically across ecotypes (LM, ecotype x sex: $F_{2, 175} = 8.29$, $p < 0.001$), with the largest size difference between sexes in anadromous fish and the smallest in resident fish (Figure 4.3). The probability of spawning decreased with increasing body size differences between males and females, but this effect had large error margins and was not significant in our model (binomial GLMM: $LR_1 = 1.24$, $p = 0.26$, Figure 4.3).

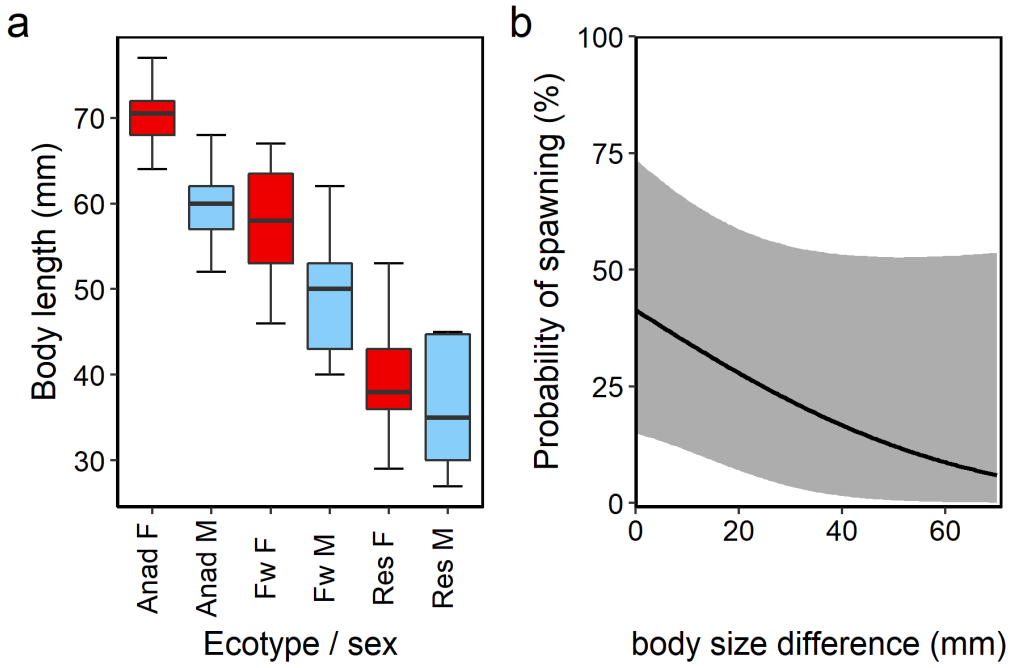

Figure 4.3 Body size differences in mate choice experiments. (a) Differences in body length between ecotypes and sexes. Anad: anadromous, Fw: freshwater, Res: resident, females are represented by red boxplots and males by blue. (b) Predicted probability of spawning with increasing differences in body size from a binomial generalised linear mixed model with year, absolute size difference, female ecotype, and whether or not male and female ecotypes were the same as predictor variables and individual male as a random effect. Bootstrapped 95% confidence intervals are shown by the grey ribbon.

4.4.2 Character displacement in body size across lochs

In all six species-pairs lochs, anadromous fish were considerably larger than resident fish (LM: $F_{1,\ 1552} = 10123.92$, $p < 0.0001$), but the mean body length of both ecotypes differed across lochs (LM: $F_{5,\ 1552} = 30.05$, $p < 0.0001$). The difference in body size between ecotypes was approximately consistent across lochs (LM, ecotype x loch: $F_{5,\ 1547} = 1.93$, $p = 0.09$), with anadromous fish tending to be larger where resident fish were also larger (Figure 4.4). Thus, there was a strong correlation between the body size of resident and anadromous fish across lochs ($r = 0.93$, $t = 4.89$, $df = 4$, $p = 0.008$, Figure 4.4).

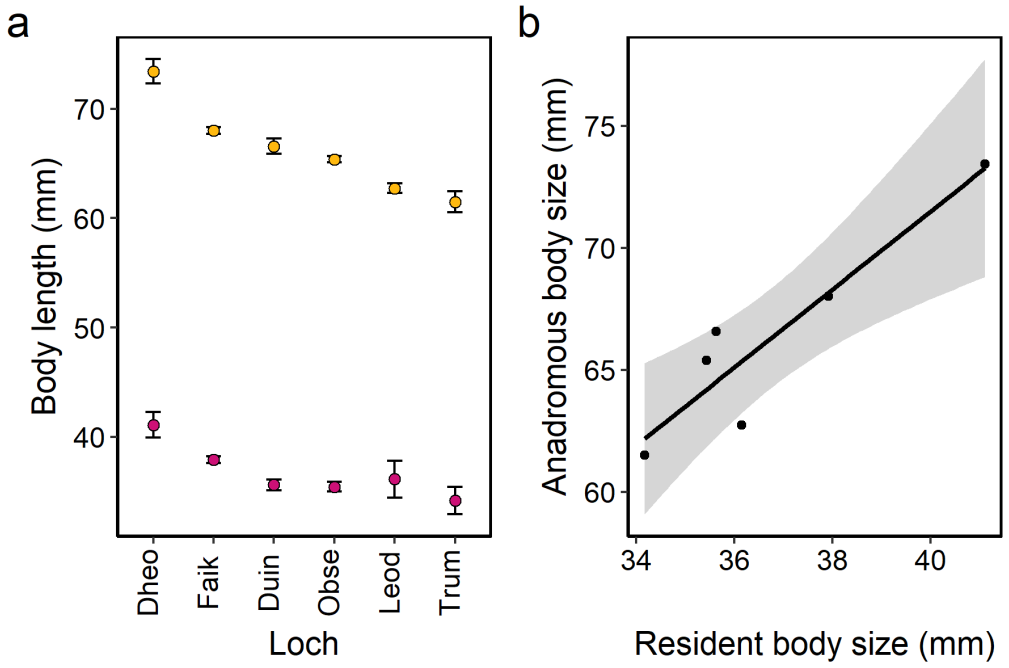

Figure 4.4 Body length variation between ecotypes across lochs. (a) Differences in body length between ecotypes across six lochs containing resident-anadromous species-pairs. Yellow circles represent the mean body length for anadromous stickleback and pink circles show the same for resident stickleback. Error bars depict the standard error of the mean (*SEM*). (b) Correlation in body length between resident and anadromous populations across lochs (Pearson's correlation coefficient: $r = 0.93$), the grey ribbon represents the standard error of a LM fit.

4.5 Discussion

We have shown that resident and anadromous stickleback display positive assortative mating, a common trait in taxa comprised of multiple ecotypes (Hollander *et al.*, 2005; Jarvis *et al.*, 2017; Machado-Schiaffino *et al.*, 2017). We found no indication, however, that assortative mating preferences were exaggerated in sympatric resident-anadromous species-pairs, compared with those of a nearby allopatric freshwater population. Our results are, therefore, inconsistent with a role for reinforcement of mate choice behaviour in driving assortative mating and maintaining RI in resident-anadromous species-pairs.

Instead, they imply that assortative mating has arisen as a by-product of adaptive divergence, rather than being explicitly selected for (Vines and Schluter, 2006).

4.5.1 By-product vs assortative mating

Allopatric populations cannot produce hybrids, so by default, any assortative mating that exists between totally isolated populations can only have evolved via the by-product mechanism (Schluter, 2001). We detected assortative mating in both allopatric and sympatric stickleback populations, which illustrates that it has evolved at least partially as a by-product of other adaptations in this system. Given the substantial phenotypic adaptations, including differences in body armour, size and shape (Chapter 2, Campbell, 1979; MacColl *et al.*, 2013; Magalhaes *et al.*, 2016), across stickleback populations on North Uist, some by-product assortative mating would be expected (Dodd, 1989; Kilias *et al.*, 1980). This mechanism is also implicated in causing assortative mating in other reproductively isolated, allopatric stickleback populations (Vines and Schluter, 2006), which suggests that reasonably substantial assortative mating, that is purely a by-product of other differences, may be common among stickleback.

Our failure to detect stronger assortative mating in sympatric populations, compared to the 'by-product' level in allopatric stickleback, suggests that assortative mating preferences are not reinforced in North Uist species-pairs. Reinforcement of mating preferences has been implicated in maintaining RI in most other known stickleback species-pairs (Gow *et al.*, 2006; Hay and McPhail, 1975; Ishikawa and Mori, 2000), and thus, our finding that it does not appear to be operating in Uist species-pairs is novel, and demonstrates that the processes maintaining RI in sympatric stickleback may not always be replicated across

independent pairs. The lack of reinforcement on mating preferences in our experiments is consistent with at least two possible scenarios. Firstly, it is possible that all assortative mating is accounted for by the by-product mechanism, and we did not detect reinforcement because assortative mating is not adaptive in this system, and therefore it does not occur (Vines and Schluter, 2006). Alternatively, reinforcement may still be involved in maintaining RI, but we failed to detect it in our mate choice experiments because it acts on mating characteristics other than female mate choice.

Our results indirectly implied an adaptive role for assortative mating because we showed that spawning occurs between resident and anadromous ecotypes, albeit infrequently, yet adult F1 hybrids are almost totally absent in wild populations (Chapter 2). It is possible that the lack of choice in mating partners in our trials forced mating between fish that would not have spawned in a natural setting (Dougherty and Shuker, 2015), but this is probably unlikely in female stickleback (Vines and Schluter, 2006), and is particularly unlikely here given the relatively low overall spawning rate in our trials (26%). The low detection rate of adult F1 hybrids in the wild is, thus, probably indicative of other reproductive barriers, possibly including a low hybrid survival rate in wild populations. This would implicate post-mating isolation in maintaining RI in this system, as is the case in other examples of strong RI in stickleback (Honma *et al.*, 1986; Kitano *et al.*, 2007; McKinnon and Rundle, 2002), and suggests that hybrids do suffer reduced fitness, which would mean assortative mating should be adaptive, and begs the question why is there not reinforcement in this system. It may be that there has not been sufficient time for reinforcement to evolve, but more likely, as well as by-product assortative mating based on mating

preferences, further assortative mating that is adaptive does occur in the wild, but is driven by factors other than direct mate choice e.g. temporal or spatial differences in spawning (Bolnick *et al.*, 2015; Filchak *et al.*, 2000; Hagen, 1967; Kume *et al.*, 2005; Sirkia *et al.*, 2018).

Spatial and temporal differences in reproductive strategy can contribute to assortative mating and reproductive isolation indirectly, by minimizing contact between closely related species or ecotypes (Borzee *et al.*, 2016; Hagen, 1967; Pegoraro *et al.*, 2016; Snowberg and Bolnick, 2012). In our experiments, males of different ecotypes differed in the substrate on which they preferred to construct their nests. Freshwater and anadromous males were somewhat similar, with the preferred substrate being sand, followed by gravel for both ecotypes. Resident males, however, almost always chose to build their nest directly on weed, a trait which has previously been described in male stickleback with a low lateral plate phenotype (Hagen, 1967; Kynard, 1979), and may be related to increased advantages of nesting in concealment. As freshwater males, which also have a low lateral plate number, were not given the option of nesting directly on weed, it is not possible to identify whether they would have shared this preference. The substantial difference in male nest location between sympatric ecotypes in both species-pair lochs implies that there could be spatial segregation between ecotypes during spawning, which could cause additional assortative mating and could also be reinforced. Ecological, rather than behavioural pre-mating isolation mechanisms may therefore be important for maintaining isolation in sympatric stickleback species-pairs.

Although cross-ecotype spawning occurred in our experiments, anadromous females and resident males never spawned in any of our trials, tentatively indicating that there may be asymmetry in mating preferences between ecotypes. This phenomenon occurs in other resident-anadromous stickleback populations (Furin *et al.*, 2012), and is partially responsible for maintaining complete RI in Japanese stickleback species-pairs (Kitano *et al.*, 2007). However, the low propensity for resident males to make nests meant that only a small number of trials with this pairing could be attempted. More trials to specifically test for asymmetrical mating preferences would provide an interesting avenue for further research.

Although we failed to detect reinforcement in mating preferences, our study did reveal behavioural differences in spawning between sympatric and allopatric populations. Females of both sympatric ecotypes were less likely to spawn at all than females from the allopatric population suggesting that sympatric populations may be generally more cautious when it comes to spawning. Population level differences in spawning propensity may be related to differences in the ability of different populations to adapt to an unnatural experimental environment (Nagel and Schluter, 1998), or could indicate that the artificial environment provided for mate choice tests in this experiment was somehow better suited to the allopatric population. Regardless of the cause, they imply behavioural differences that could have come about as a result of sympatric coexistence.

4.5.2 Evidence for isolation based on body size

We found that across six lochs containing species-pairs, the body size of anadromous fish was strongly correlated with the body size of resident fish. This pattern strongly suggests structure in the anadromous population, with individuals having strong natal homing tendencies, as is evident in certain other anadromous species (Engstedt *et al.*, 2014; Nordeng, 2009). There are at least three possible explanations for the variation in size between lagoons, which are not mutually exclusive: (i) within ecotype adaptation to local conditions has led to parallel evolution (or plasticity) of size differences across lagoons with varying ecological conditions (MacColl *et al.*, 2013), but this seems unlikely given the small amount of time spent by anadromous fish in lagoons: probably two to three months post hatching (McPhail, 2007), before migrating to the sea, where conditions are probably relatively uniform. (ii) A low level of gene flow between sympatric populations could result in the flow of body size determining genes from locally adapted resident fish into sympatric anadromous populations causing parallelism in size. Or (iii), recurrent character displacement has meant that selection has repeatedly maintained the mean size of resident and anadromous ecotypes at a similar distance from one another. This could be generated by either ecological or reproductive factors, the latter of which would be consistent with reinforcement acting on these populations.

Although the species-pairs in our mate choice experiments differed substantially in size, and we were able to detect assortative mating, we found no evidence that it was related to body size, which was somewhat unexpected as size assortative mating is extremely common in stickleback (Boughman *et al.*, 2005; McKinnon

et al., 2004; Nagel and Schluter, 1998). This suggests that reproductive character displacement based on body size is not responsible for the correlation in size between resident and anadromous stickleback across lochs. Ecological character displacement is still possible, but again, seems unlikely because of the limited time anadromous fish spend in the lochs (McPhail, 2007). Further investigation into both the differences in, and potential effects of differences in environmental conditions across lochs, and the possibility of gene flow in sympatric pairs would, therefore, be useful to draw conclusions about the causes of the body size correlations found here.

Aside from body size, there are many characteristics, such as nest size and shape (Rushbrook *et al.*, 2008), nesting material (Ostlund-Nilsson and Holmlund, 2003), nest microhabitat (Bolnick *et al.*, 2015; Hagen, 1967), male courtship behaviours (Ridgway and McPhail, 1984) and male nuptial colouration (Milinski and Bakker, 1990), that may be involved in female mate choice in stickleback, and consequently assortative mating, if these factors differ consistently between ecotypes (Bakker, 1993; Kunzler and Bakker, 2001). To identify the traits which underlie the assortative mating in Uist stickleback populations, further mate-choice trials to characterise variation in these other factors and their relationship with female mate choice would be necessary.

4.5.3 Conclusions

We demonstrated that assortative mating occurs among freshwater, resident and anadromous ecotypes on North Uist, but found little evidence that this was driven by differences in body size or that reinforcement was operating on these mate choice characteristics in sympatric resident-anadromous species-pairs. Our

investigation, therefore, highlights the importance of accounting for non-adaptive explanations of assortative mating when testing for signatures of reinforcement (Servedio, 2004; Vines and Schluter, 2006), and suggests that either it is not important in this system, or it operates on traits other than direct mating preferences. Other pre and postzygotic mechanisms, such as differences in preferred nesting microhabitat, and potential genetic incompatibilities are, therefore, probably also functioning to maintain RI in stickleback species-pairs and merit further investigation. The strongly isolated species-pairs on North Uist, which lack signatures of reinforcement in mating preferences provide a valuable resource for further analysis of the ecological and genetic characteristics which underlie patterns of speciation in stickleback.

CHAPTER 5 : PREADAPTATIONS TO OVOVIVIPARITY – INTERNAL FERTILISATION AND EMBRYONIC DEVELOPMENT IN THE THREE-SPINED STICKLEBACK, A NON-COPULATORY, OVIPAROUS SPEICES

5.1 Abstract

The transition from direct egg-laying to retaining and giving birth to live young has a strong claim to being a major transition in the history of life. Despite its repeated evolution across the fishes, there are only two existing records of normally egg-laying fish species retaining embryos within the ovaries. Here we report the discovery of well-developed embryos in the ovaries of a female three-spined stickleback (*Gasterosteus aculeatus*), a non-copulatory, normally oviparous species. Extracted from the parent fish, these embryos hatched and 57 fry grew to adulthood in the lab. The parent fish was genetically female, and possessed no testicular tissue, while the offspring were a mixture of males and females and carried multiple alleles that were absent in the parent fish. This confirms that the embryos did not develop parthenogenetically, nor were they a result of hermaphroditic self-fertilisation, but rather must have been the result of internal fertilisation from a male stickleback. The development of this phenomenon, which is extremely rare, may have been facilitated in this population by an unusual tendency for females to become egg-bound. It indicates that the ovaries of female stickleback provide a suitable environment for embryonic development, almost to the stage of hatching, and suggests that some major transitions may arise almost spontaneously.

5.2 Introduction

The evolution of complex new phenotypes involving multifaceted and often synergistic changes in many different traits, has been used as an argument against Darwinian gradualist evolution, on the basis that incomplete transitions cannot be functional (Mivart, 1871). Counter arguments have pointed out that intermediate structures, or 'preadaptations', may arise randomly by chance, as non-adaptive by-products of other adaptations, or initially evolve to serve a different function, but by retrospective good fortune come to be useful in their current role (Bock, 1959; Bock and Vonwahlert, 1965; Gould, 1980; Prum and Brush, 2002). Nevertheless, partially developed major transitions are seldom observed (Koonin, 2007; Maynard Smith and Szathmary, 1995). Females in the great majority of animal species reproduce oviparously, laying eggs that develop and hatch in the external environment (Blackburn, 1999). The transition from oviparity to the retention of developing embryos internally, either as eggs (ovoviviparity), or embryos nourished by a placenta (viviparity), and giving birth to live young, has a strong claim to being a major transition in the history of life (Blackburn, 1999; Wourms and Lombardi, 1992). It involves multiple interrelated changes in physiology and behaviour, and potentially evolves as a response to unfavourable environmental conditions (Goodwin *et al.*, 2002; Shine and Bull, 1979).

Live-bearing has evolved independently in vertebrates over 160 times (Blackburn, 1999), but its distribution is highly uneven across taxa. It is almost ubiquitous among mammals (Sharman, 1976), non-existent in birds (Blackburn and Evans, 1986), and occurs in some reptiles, amphibians and fish (Blackburn,

1999). In mammals, it probably had a single evolutionary origin, but has evolved independently over 100 times in squamate reptiles, five times in amphibians and at least 35 times in fishes (Blackburn, 1999). Despite the repeated evolution of ovoviviparity across fish lineages, the huge number of extant egg-laying species, and the colossal number of these that must have been examined by fish biologists, records of normally non-copulatory, oviparous fish retaining developing embryos, a potential precursor to the evolution of live-bearing, are incredibly rare. There are only two previous records of this phenomenon. Multiple specimens of *Hemilepidotus gilberti*, were discovered with eyed embryos in the ovaries of spent females, but all embryos either died early in development or possessed significant physical deformities, and would not have survived post-hatching (Hayakawa and Munehara, 2001). A single three-spined stickleback (*Gasterosteus aculeatus*, hereafter 'stickleback') was also found with well-developed embryos retained in the ovaries (Greenbank and Nelson, 1959), but these embryos were not assessed for deformities or signs of life. Here we document in detail a second occurrence of this phenomenon in stickleback, in which embryos, once removed from the mother, hatched and grew to adulthood with no physical abnormalities.

Stickleback are a non-copulatory, oviparous, sexually reproducing species, with a simple XY sex-determining system (Peichel *et al.*, 2004). Female stickleback are batch spawners, retaining each clutch of ovulated eggs in the ovaries prior to spawning (Roufidou *et al.*, 2018). If females fail to either spawn or spontaneously release their eggs, the eggs can become overripe and harden in the reproductive tract, obstructing the release of further clutches and preventing spawning (Bromage *et al.*, 1992; Springate *et al.*, 1984). Retained eggs are lost

for reproduction (Lam *et al.*, 1978), and egg-bound females ultimately die from their condition (Hansen *et al.*, 2016), but, despite this, egg-bound females can be reasonably common in some stickleback populations (Borg and Vanveen, 1982; Lam *et al.*, 1978; Roufidou *et al.*, 2016). The prolonged retention of overripe ovules is considered a non-adaptive 'accident' (Bromage *et al.*, 1992; Hansen *et al.*, 2016), caused by unfavourable population densities, sex ratios, environmental conditions or the presence of pollutants, reviewed in Rideout *et al.* (2005). However, the retention of ovules is necessary for internal fertilisation, which is an essential step in the transition from oviparity to live-bearing (Wourms and Lombardi, 1992), and thus a tendency to retain ovules could be considered a preadaptation to this transition.

We discovered an egg-bound marine stickleback in a saline coastal lagoon on North Uist, Scottish Western Isles with ovaries containing a clutch of well developed, eyed embryos. This phenomenon has at least three possible explanations: (i) the ova were not fertilised, but developed by parthenogenesis, i.e. by artificial stimulation by heat, chemical or physical shock, in the absence of sperm. Parthenogenesis is common in some animal species (Simon *et al.*, 2003), and gynogenesis (development in which the embryo contains only maternal chromosomes due to activation of an egg by a sperm that degenerates without fusing with the egg nucleus) occurs in some fish (Beukeboom and Vrijenhoek, 1998). Gynogenesis has been stimulated artificially in stickleback (Samonte-Padilla *et al.*, 2011), but neither gynogenesis, nor true parthenogenesis have been recorded in natural stickleback populations. (ii) The stickleback may have been hermaphroditic, containing both male and female reproductive organs, and the eggs could thus have been self-fertilised internally, with sperm

produced by the same individual. Hermaphroditism can be relatively common in certain fish species (Devlin and Nagahama, 2002; Harrington, 1961; Warner, 1984), and can be chemically induced in stickleback (Bernhardt *et al.*, 2006). Furthermore, an apparently hermaphroditic population of stickleback exists in Alaska, from which the single other individual carrying developing embryos was collected (Greenbank and Nelson, 1959), making hermaphroditism an apparently strong possibility. (iii) Finally, eggs could have been internally fertilised by the sperm of another stickleback, which entered the reproductive tract from the external environment. This was deemed to be the case in *H. gilberti* (Hayakawa and Munehara, 2001), however, the spawning behaviour in this species is such that females release eggs very slowly and males emit sperm onto the egg mass several times, after which females remain on the egg mass for several minutes (Hayakawa and Munehara, 1996), likely allowing the transfer of sperm into the female reproductive tract. In contrast, female stickleback lay their eggs and exit the nest before the male follows and releases sperm onto the eggs (Barber *et al.*, 2006), making the transfer of sperm into the reproductive tract of female stickleback much less likely.

Our three hypotheses for the occurrence of fertilised embryos inside a female stickleback make clearly distinct, testable predictions about the genetic makeup and gonadal structure of the parent fish and the genetic relationship between parent and offspring. (i) If the eggs were not fertilised, and developed by parthenogenesis, the parent fish would be genetically female, possess ovaries but no testes, and the offspring would be genetically identical to the single parent fish. (ii) If the parent was hermaphroditic, and eggs were fertilised by sperm produced by the same individual, the parent fish would have both ovarian and

testicular tissue in the gonads, the offspring may be genetically male or female, but could only carry alleles that were also present in the parent fish. (iii) If the eggs were fertilised by the entry of sperm from another stickleback into the reproductive tract, the parent fish would be genetically female and possess only ovarian tissue in the gonads. The offspring would probably be a mix of genetically male and female individuals and would carry alleles from the father that were not present in the mother. We carried out a detailed examination of the gonads of the parent fish, and genetic sex testing alongside analysis of the allelic composition of the parent and offspring using a series of microsatellite markers to test these three hypotheses. To examine the possibility that the presence of the embryos may be related to a tendency by fish in the same population to retain eggs, we compared the prevalence of egg-bound gravid females across stickleback populations on North Uist.

5.3 Methods

5.3.1 Fish collection, dissection and egg husbandry

As part of a programme to breed fish to raise in the lab, stickleback were caught using un-baited minnow traps (Gee traps, Dynamic Aqua, Vancouver). Traps were set overnight in Faik (57°38'N; 7°12'W), a brackish coastal lagoon, in water 30-100cm deep and lifted the following morning. On 27th April 2015 a gravid female anadromous fish, displaying the classic 'berried' abdomen of an egg-bound fish (Lam *et al.*, 1978), was caught. The fish was transported back to the laboratory in aeriated loch water. On arrival the fish was euthanized by overdose of MS222 anaesthesia, followed by destruction of the brain, in accordance with Schedule One of the UK Home Office regulations. This was followed by

immediate dissection by cutting along the right flank, from the cloaca to the dorsal margin of the operculum. Eggs containing developing embryos were identified and the ovaries and the rest of the body cavity were thoroughly examined for the presence of testicular tissue before being preserved in 70% ethanol.

Eggs which contained developing embryos were removed from the ovaries and kept in 1% saline solution before being examined to record stages of development, potential abnormalities and signs of life (e.g. the presence of a heart beat) using a light microscope. Eggs containing live embryos were transported, chilled, in saline solution to aquaria at the University of Nottingham, where they were maintained in strongly oxygenated water containing 1% marine salt. Between four and seven days following the removal of developing embryos from the ovaries, 57 fry hatched successfully and were raised to adulthood in the lab, on a diet of live brine shrimp and defrosted, washed bloodworm, fed daily.

5.3.2 Sectioning and examination of gonads

The gonads of the parent fish, along with those of six other individuals (three of which were genetically and morphologically determined to be male, and three female) from a nearby loch (Chru, 57°35'37"N; 7°11'44"W), were rehydrated by the sequential dilution of ethanol from 70% to 0%, using deionised water. Gonadal tissues were then fixed in 10% neutral buffered formalin, dehydrated in graded alcohol-xylene and embedded in paraffin wax using a Leica TP1020 Tissue Processor. Sections of tissue (8µm thick) were taken using a Slee Cut 4060 Microtome and stained using Haematoxylin-Eosin before being mounted on glass microscope slides using DPX. The sections of gonadal tissue from the

parent fish were compared to those from the three known male and female stickleback and examined thoroughly for the presence of testicular tissue by three independent individuals.

5.3.3 Sample collection and DNA extraction

DNA was collected from the parent fish and 56 of 57 offspring. DNA was collected by running a single sterile swab (Sarstedt cotton Forensic Swabs) repeatedly over both flanks of each fish. Swab heads were removed and placed on ice in individual Eppendorf tubes prior to DNA extraction. DNA was extracted using Viagen DirectPCR Lysis Reagent (Mouse Tail), following the manufacturers standard protocol with the following modifications. 270µl of DirectPCR Lysis Reagent and 30µl proteinase K were added to each swab, ensuring the swab head was completely covered by the solution. Samples were incubated for 5 hours at 55ºC whilst rotating at 250rpm. The swab head was removed from each tube, and the solution then incubated at 85ºC for 45 minutes before cooling to room temperature.

5.3.4 Genetic sex determination and microsatellite analysis

Sex testing was performed on the parent fish and 56 adult offspring. Sex determination was conducted by PCR amplification of three loci (*Idh*, *Gasm6* and *Stn190*, Table 5.1), which, combined, produce an accurate determination of sex (Toli *et al.*, 2016). DNA samples extracted from an adult male and female in breeding condition were used as positive controls. To further assess whether a second individual contributed to the genetic make-up of the offspring three additional sets of microsatellite loci (*Stn57*, *Stn201* and *Stn317*, Table 5.1),

which are polymorphic in other stickleback populations on North Uist (El Nagar, 2014), were analysed for the parent fish and 14 randomly selected offspring.

Table 5.1 PCR primers for sex determination and microsatellite analysis Primer sequences used for DNA amplification in stickleback sex determination and microsatellite analysis, and the researchers by whom they were developed. Primers are identified by the name of the loci followed by 'F' for forward primers and 'R' for reverse primers.

Primer	Sequence	Source
ldh_F	5' GGGACGAGCAAGATTTATTG 3'	(Peichel *et al.*, 2004)
ldh_R	5' TTATCGTTAGCCAGGAGATGG 3'	(Peichel *et al.*, 2004)
Gasm6_F	5' GATTAAAGGAACCAGAGGGG 3'	(Natri *et al.*, 2013)
Gasm6_R	5' GGAACTGGAATTTTGAGGGT 3'	(Natri *et al.*, 2013)
Stn190_F	5' CGATAATGCACGACAATCTCC 3'	(Peichel *et al.*, 2001)
Stn190_R	5' AACACATGAGCGTTATGGC 3'	(Peichel *et al.*, 2001)
Stn57_F	5' GATGGTGCCCATAAGACTCG 3'	(Peichel *et al.*, 2001)
Stn57_R	5' CATGTGTGGATGAAGGATGC 3'	(Peichel *et al.*, 2001)
Stn201_F	5' TCACTTCACAGGGACAATGG 3'	(Peichel *et al.*, 2001)
Stn201_R	5' ACTGCTGGAGGATGAAATGG 3'	(Peichel *et al.*, 2001)
Stn317_F	5' CAGGATGAAATGAAGGTCTGG 3'	(Peichel *et al.*, 2001)
Stn317_R	5' TGTGGACTTTCAGATGAGCG 3'	(Peichel *et al.*, 2001)

All PCR reactions were carried out in 20µl volumes containing 6µl nuclease-free H_20, 10µL 2X Biomix^{TM} red reaction mix (Bioline), 1µl of both forward and reverse primers (10µM) and 2µl of template DNA (approximately 20ng). For all PCRs, thermocycling was set up as follows: Initial denaturation at 96°C for two minutes, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 70°C for 30 seconds, followed by a final extension at 70°C for two minutes. To score genotypes, PCR products were electrophoresed on a 4% agarose gel run at 100V for one hour, alongside 100bp DNA ladder. For sex testing primers the presence of a single band indicated a female and the presence of a double band indicated a male. On the few occasions where the result of one primer pair differed from the others, the

sex of the fish was assumed to be that of which two out of the three pairs of primers agreed. For all six microsatellite loci the occurrence of alleles in the offspring that are not present in the mother indicated that a second individual contributed to the genetic make-up of the offspring.

5.3.5 Prevalence of egg-bound females across populations

During April-May of 2007, 2008, 2010 and 2011, 230 gravid female stickleback were collected from 24 populations, across 22 lochs on the island of North Uist, Scottish Western Isles (Table 5.2). Females were euthanized by overdose of MS222 anaesthesia, followed by destruction of the brain, in accordance with Schedule One of the UK Home Office regulations. They were then measured for standard length (from the tip of the snout to the tip of the caudal peduncle) and visually assessed for the 'berried' abdominal appearance typical of egg-bound fish, before being dissected. The ovaries were removed and all eggs were counted and assessed for signs of being overripe, e.g. larger size, increased transparency, cytoplasmic aggregation and the accumulation of oil droplets at the animal pole (Lam *et al.*, 1978).

Table 5.2 Stickleback sampling locations Lochs on North Uist, Scottish Western Isles, from which gravid female stickleback were collected. N = total sample size of gravid stickleback. Location is given by latitude followed by longitude.

Loch ID	Population	N	Salinity	Location
Abma	Resident	6	Freshwater	57°38'37"N; 7°21'55"W
Acha	Resident	1	Freshwater	57°35'39"N; 7°23'18"W
Aroi	Resident	2	Freshwater	57°35'40"N; 7°25'52"W
Bhar	Resident	11	Freshwater	57°34'16"N; 7°18'8"W
Bhei	Resident	4	Freshwater	57°37'46"N; 7°13'45"W
Buai	Resident	19	Freshwater	57°38′48"N; 7°11′48"W
Chru	Resident	7	Freshwater	57°35'37"N; 7°11'44"W
Daim	Resident	9	Freshwater	57°35'32"N; 7°12'30"W
Dubh	Resident	6	Freshwater	57°34′54"N; 7°24′12"W
Eisi	Resident	3	Freshwater	57°37'50"N; 7°21'8"W
Faik	Resident	12	Brackish	57°38'7"N; 7°12'54"W
	Anadromous	10		
Fhai	Resident	12	Freshwater	57°34'6"N; 7°22'45"W
Gill	Resident	9	Freshwater	57°36'4"N; °24'37"W
Host	Resident	21	Freshwater	57°37′40"N; 7°29′18"W
Leod	Anadromous	2	Brackish	57°32'39"N; 7°20'8"W
Maga	Resident	12	Freshwater	57°36′10"N; 7°28′54"W
Maig	Resident	12	Freshwater	57°35'44"N; 7°12'15"W
Mora	Resident	5	Freshwater	57°34'29"N; 7°16'31"W
Obse	Resident	2	Brackish	57°36'6"N; 7°10'22"W
	Anadromous	9		
Reiv	Resident	14	Freshwater	57°36'39"N; 7°30'50"W
Scad	Resident	18	Freshwater	57°35′6"N; 7°14′10"W
Torm	Resident	24	Freshwater	57°33'46"N; 7°19'1"W

5.3.6 Statistical analysis

All statistical analyses were carried out in R version 3.1.4 (R.Core.Team, 2017). To test for a correlation between body length and number of eggs in normally gravid females, both variables were log transformed and a t-test based on Pearson's correlation was used. Because of the significant increase in the number of eggs relative to body length in egg-bound females, all females that were identified as egg-bound were excluded from the correlation analysis. To test

whether the sex ratio in the offspring differed from an expected ratio of 50:50, and to test for differences in the prevalence of egg-bound females in the anadromous population in Faik, compared with all other populations sampled on North Uist combined (Table 5.2), chi-squared tests were used.

5.4 Results

5.4.1 Analysis of gonads

Initial examination of the parent fish identified ovaries containing two clutches of eggs, the oldest (proximal to the cloaca) of which contained many developing embryos (Figure 5.1). Embryos, which were well-developed and had reached the stage of eyeing and body pigmentation [stage 21 of 24 stages of pre-hatching embryonic development in stickleback (Swarup, 1958)] were distributed throughout the older clutch, even to its distal margin from the cloaca (Figure 5.1). The viability of embryos was confirmed by the observation of a heartbeat, and no physical abnormalities, or deformities, were observed on inspection of the eggs. There was no testicular tissue evident anywhere on the ovaries or in the body cavity of the parent fish. On further inspection of detailed, stained cross sections of the ovaries, all three independent researchers agreed that that they contained developing oocytes only, with no testicular tissue present (Figure 5.2).

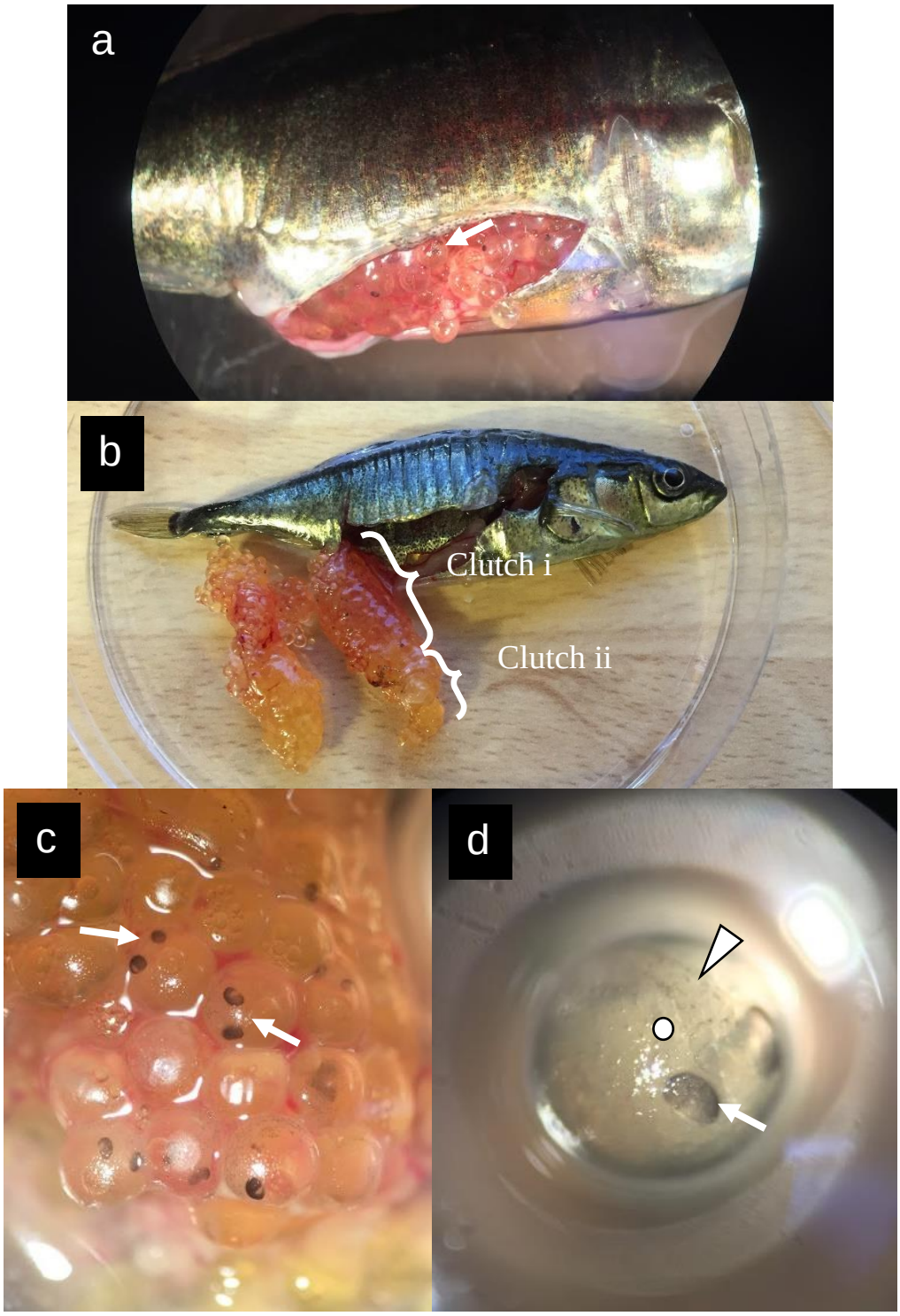

Figure 5.1 Examples of internally developed embryos. (a) The abdomen of a three-spined stickleback female with ovaries containing developing embryos. (b) The parent fish with ovaries removed and the two developing clutches of eggs in the ovaries labelled. (c) A cluster of the developing embryos removed from the ovary, and (d) a single embryo removed from the ovary. Images show embryos with developed eyespots (→), spinal cord (►) and heart (●), which was observed beating under a microscope.

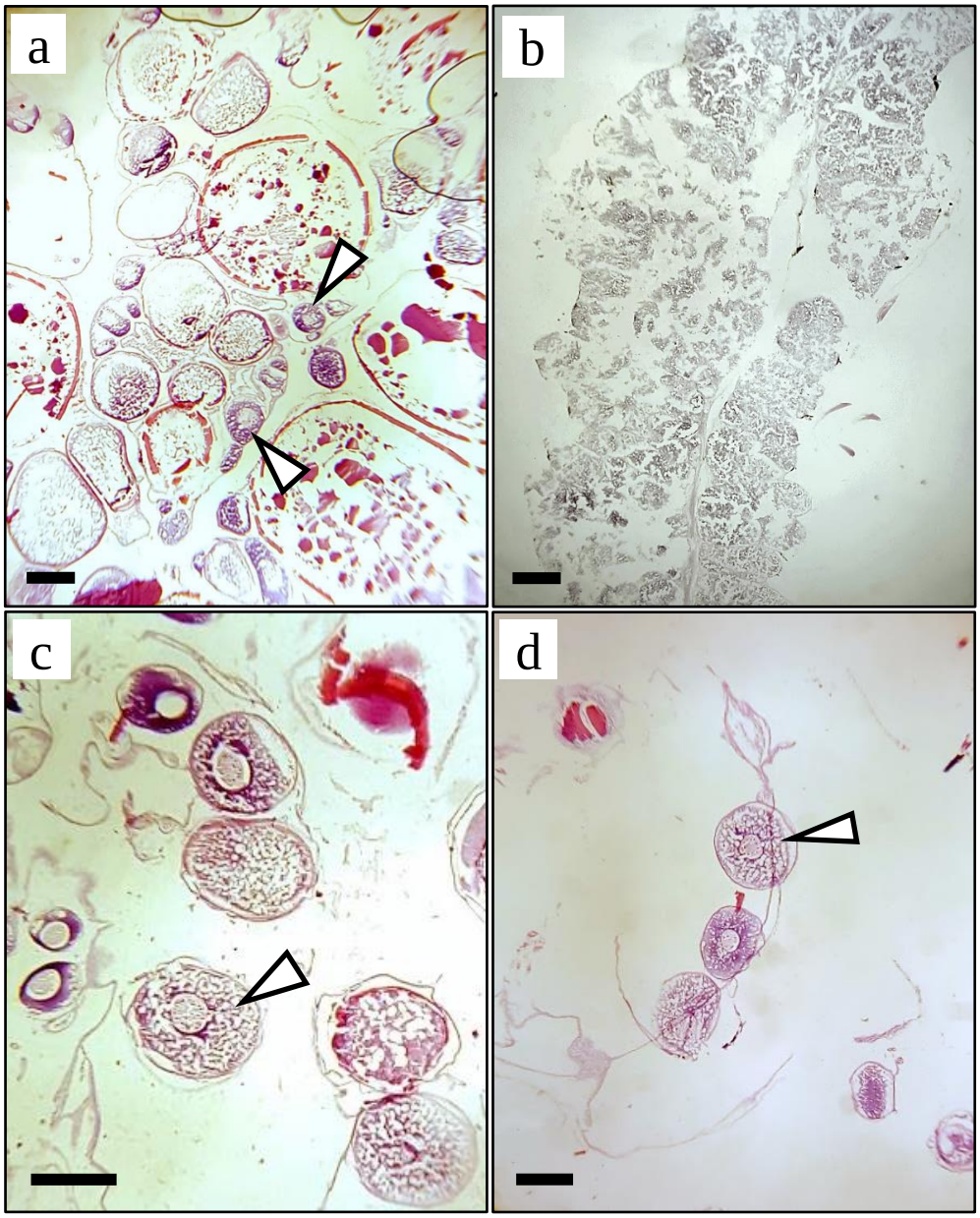

Figure 5.2 Cross sections of gonadal tissue. Cross sections of (a) ovarian and (b) testicular tissue of confirmed genetic female and male three-spined stickleback respectively. (c & d) Cross sections of the gonadal tissue of the parent fish carrying developed embryos. Black bars indicate 200µm, arrows show mature oocytes post vitellogenesis.

5.4.2 Sex determination and microsatellites

Microsatellite analysis revealed that the offspring were not genetically identical to the parent fish, or to one another (Table 5.3). The parent fish was homozygous at four of the six microsatellite loci, while at least some of the offspring were heterozygous at five of the six loci (Table 5.3) Of the 56 offspring, 19 possessed at least one allele that was not present in the parent fish.

The parent fish was determined to be genetically female by all three sex determining loci (Table 5.3), while both sexes were present in the offspring (Table 5.3), but there were more females (75%, 42/56 offspring) than males (25%, 14/56 offspring, $X^2 = 14$, $df = 1$, $p < 0.001$).

Table 5.3 Genotypes for the parent fish and offspring at six microsatellite loci. Counts of individuals who were homozygous for the smaller microsatellite alleles: Homo (1, 1), heterozygous: Hetero (1, 2), or homozygous for the larger microsatellite alleles: Homo (2, 2) are shown for each microsatellite loci. For the three sex determining loci (*Idh, Gasm6* and *Stn190*), heterozygosity indicates males.

Marker	Parent fish			Offspring		
	Homo (1, 1)	**Hetero (1, 2)**	**Homo (2, 2)**	**Homo (1, 1)**	**Hetero (1, 2)**	**Homo (2, 2)**
Idh	0	0	1	0	15	41
Gasm6	0	0	1	0	14	42
Stn190	1	0	0	0	13	43
Stn57	0	1	0	5	5	4
Stn201	0	1	0	6	5	3
Stn317	1	0	0	14	0	0

5.4.3 Prevalence of egg retention across populations

There was a strong correlation between body length and the number of eggs in the ovaries of normally gravid females (r = 0.91, t = 32.24, df = 226, $p < 0.0001$, Figure 5.3). Egg-bound females, which were found only in Faik, the same population as the female with embryos, had almost twice the number of eggs in their ovaries than normally gravid females of a similar size (Figure 5.3). Of the 10 gravid anadromous females in Faik, two were egg-bound (20%), while none of the 208 gravid females from 23 other populations on North Uist were egg-bound ($\chi^2 = 24.22$, $df = 1$, $p < 0.0001$, Figure 5.3).

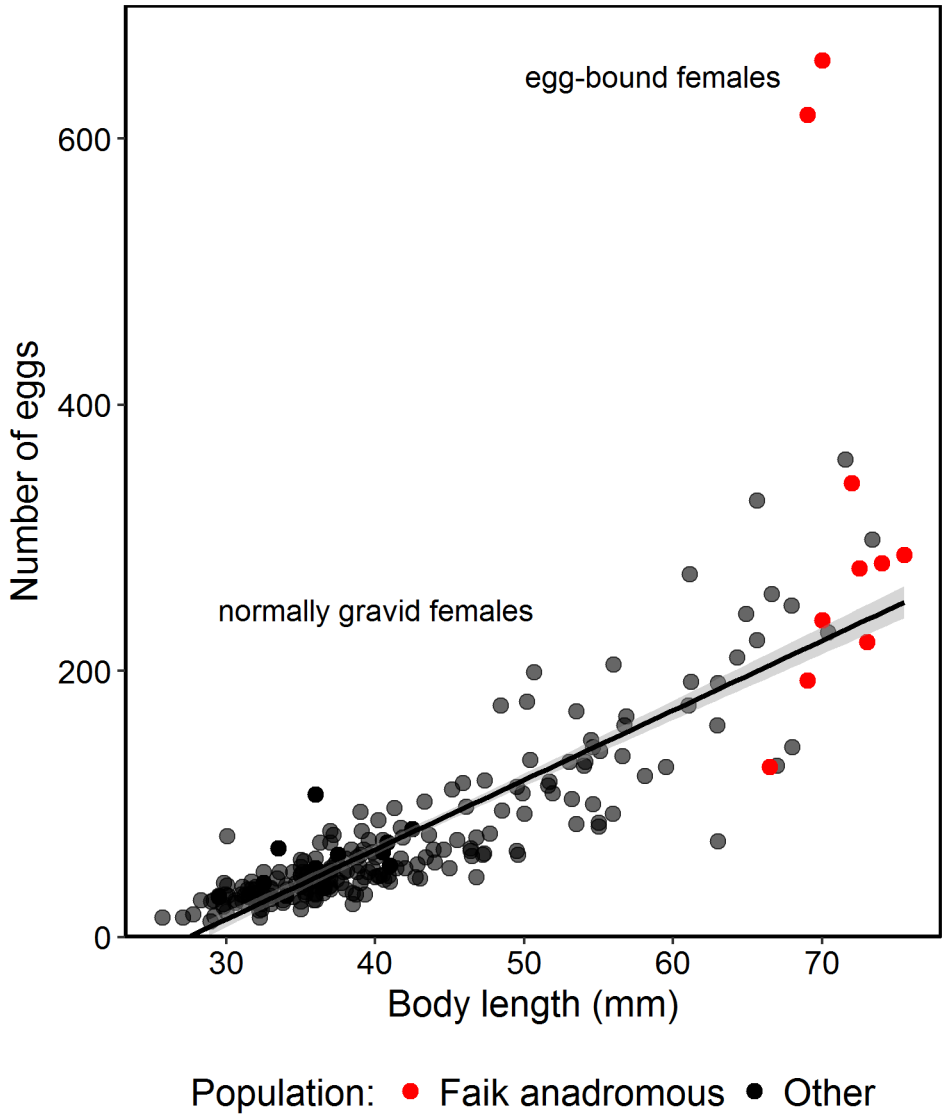

Figure 5.3 Relationship between body length and number of eggs. Correlation between body length and the number of eggs inside the ovaries of gravid females (back transformed from the log of both variables). Red circles represent gravid anadromous females from Faik, black circles show gravid females from 23 other populations across 21 lochs on North Uist (Table 5.2). The correlation between body length and the number of eggs in normally gravid females (excluding egg-bound females) is shown by the black line (Pearson's correlation coefficient r = 0.91, based on the log-log relationship) ± S.E., indicated by the grey ribbon.

5.5 Discussion

We identified a clutch of almost fully developed embryos inside the ovaries of a stickleback; a non-copulatory, oviparous species. Examination of the embryos showed that they had developed normally, inside the parent fish, to the stage of eyeing and body pigmentation, one of the final stages of pre-hatching embryonic development in stickleback (Swarup, 1958), likely corresponding to 5.5 days post-fertilisation, with no visible signs of deformity. This is contrary to the one other known case of facultative internal fertilisation in a non-copulatory fish, where a high proportion of the embryos found in the ovaries showed signs of significant deformities (Hayakawa and Munehara, 2001). We showed that the embryos removed from the parent stickleback were viable, since they hatched and grew to adulthood successfully in the lab. However, it is not possible to know whether they would have survived to hatching inside the parent fish, nor whether they would have been able to exit the ovaries of the parent, or survive in the wild without the male parental care that is normal in stickleback.

We found no evidence of testicular tissue in the gonads of the parent fish, and sex testing confirmed that she was genetically female, and therefore the embryos cannot have been produced hermaphroditically. This is particularly interesting given that hermaphroditism was implicated as the most likely explanation for the one other record of developed embryos in the ovaries of stickleback (Greenbank and Nelson, 1959). In fact, close inspection of diagrammatic representations of the internal anatomy of stickleback in Greenbank and Nelson (1959, figures 11 and 12), suggests the authors probably misidentified the internal organs and misinterpreted the normal male and female reproductive anatomy as

hermaphroditism. It appears that they interpreted the hypertrophied kidneys of male stickleback as their testes, and then compounded their error by identifying the testes as ovaries! Kidney enlargement is normal in sexually mature, adult male stickleback: it is where they synthesise the protein spiggin, which they use as glue to construct their nests (Jakobsson *et al.*, 1999; Seear *et al.*, 2015).

We also found that while the parent fish was genetically female, the offspring were a mix of genetically male and female individuals, and carried alleles for three (sex-determining) microsatellite markers that were not present in the parent fish. This means that the embryos cannot have been produced parthenogenetically, and, therefore, the eggs must have been fertilised internally by a separate, male individual. Furthermore, developing embryos were distributed throughout the older clutch of eggs, some well away from the cloaca, indicating that sperm were able to penetrate almost the entirety of the ovaries. The ovarian fluid of female stickleback greatly prolongs sperm motility, to facilitate spawning in freshwater (Elofsson *et al.*, 2003; Elofsson *et al.*, 2006), and female stickleback may prefer to spawn with males whose nests already contain the eggs of other females (Ridley and Rechten, 1981), thus, we hypothesise that sperm most likely entered the reproductive tract of the parent fish via contact with recently fertilised eggs in a nest.

The fact that the embryos had reached such an advanced stage of development indicates that the ovaries of female stickleback provide a suitable environment for embryonic development. Stickleback embryos acquire all the necessary energy for growth from the yolk provided by the mother within the egg (Wootton, 1994), and although they would not normally develop internally, are

capable of modulating their exposure to maternally derived, potentially harmful chemicals via active transport following fertilisation (Paitz *et al.*, 2016). This could explain why embryonic development was not adversely affected by the chemical / hormonal environment of the mother. However, a rich oxygen supply is necessary for proper embryonic development and male stickleback invest heavily in parental care of the eggs, including frequent fanning to increase oxygen delivery (Reebs *et al.*, 1984; Sargent and Gebler, 1980). Ovoviviparous fish specifically regulate the dissolved oxygen content in ovarian fluid according to embryonic requirements (Boehlert *et al.*, 1991), but whether this is possible in stickleback is not known. Nonetheless, the normal development of embryos inside the ovaries in this case indicates that there must have been sufficient oxygen for normal development, and that both female stickleback and their embryos already possess many of the preadaptations necessary for embryos to develop within the ovaries.

Given the extensive research that has been conducted on stickleback, a model organism (Hendry *et al.*, 2009; McKinnon and Rundle, 2002; Peichel and Marques, 2017), and oviparous fish in general, the fact that internal embryonic development has only been recorded once before in stickleback (Greenbank and Nelson, 1959), and apparently only once more across all other normally oviparous fish (Hayakawa and Munehara, 2001), suggests that it is extremely uncommon. We showed that the population to which the parent fish belonged had an increased tendency towards prolonged egg retention (20% of anadromous females in Faik were egg-bound, while no egg-bound females were found in any of the 23 other populations sampled), which indicates that location specific biotic or abiotic factors in that particular loch may make fish more prone to retaining

their eggs (Rideout *et al.*, 2005), and could increase the likelihood of accidental internal fertilisation during failed egg-laying attempts. However, egg-bound females have been observed in other populations of stickleback (Lam *et al.*, 1978; Roufidou *et al.*, 2018), and oviparous fish species (Feindel *et al.*, 2011; Hansen *et al.*, 2016; Speare, 1965; Treasurer and Holliday, 1981), with no record of internal embryonic development. Nonetheless, prolonged egg retention could be a preadaptation that facilitates the transition to internal embryonic development. We cannot know whether the occurrence of such isolated incidence of the internal retention of developing embryos indicates the nascent evolution of a transition to live-bearing, but they do suggest that such transitions are possible and can arise more or less spontaneously, on an individual level. Stickleback clearly possess many attributes or preadaptations necessary for ovoviviparity and further investigations into the reproductive physiology of female stickleback and the causes and consequences of prolonged egg retention in this species would be extremely interesting with regard to explaining this anomaly.

CHAPTER 6 : GENERAL DISCUSSION

This is an exciting time to be studying speciation. The recent recognition that speciation is a continuous process (Hendry, 2009; Hendry *et al.*, 2009; Mallet, 2008; Nosil *et al.*, 2009; Powell *et al.*, 2013) has allowed research based on incomplete speciation, and divergent ecotypes to flourish. As a consequence, our understanding of the processes and events that initiate speciation, and those which maintain divergence and facilitate movement along the speciation continuum is rapidly growing. In this thesis, I identified and characterised two distinct, morphologically and genetically isolated three-spined stickleback (*Gasterosteus aculeatus*, hereafter 'stickleback') ecotypes on North Uist, which have appeared to have progressed significantly further towards speciation than in most other locations. I then used these ecotypes to investigate the causes and contexts of the early stages of speciation. Here I review the main findings of this work, highlight their contribution to current knowledge on speciation and make suggestions for avenues opened up by this project for further research.

6.1 Thesis summary

I used morphological data on body size, shape and external bony armour to show that there are three phenotypically divergent stickleback ecotypes on North Uist. I then showed that a particularly high proportion of variation in the most important distinguishing characteristic between ecotypes, lateral plate phenotype, is explained by a single locus *Eda*, with divergent Eda^{C} and Eda^{L} alleles corresponding to divergent complete and low plated phenotypes respectively. Morphological data strongly suggested that, in a handful of locations on North Uist, pairs of resident and anadromous ecotypes co-exist in

the absence of intermediate morphs. *Eda* genotyping showed that, in these species-pair locations, the two ecotypes were fixed or approaching fixation for divergent *Eda* alleles. In contrast, in a rare location in which resident and anadromous populations met a third anomalous ecotype, both morphological and genetic separation between all three ecotypes was absent, and intermediate morphs were commonplace. Together, this suggested that hybridisation in species-pairs was extremely low (hybrids could have been produced in species-pairs but eliminated by natural selection before reaching adulthood, but the presence of apparently fully functional adult hybrids in other locations makes this highly unlikely). It also suggested that the presence of additional ecotypes, and their associated ecological consequences, may inhibit, or even reverse speciation (Chapter 2).

I then attempted to identify the geographical contexts and evolutionary mechanisms which contributed to the evolution of stickleback species-pairs on North Uist. I used a multi-disciplinary approach, utilising historic environmental information stored in lake sediments to reconstruct periods of saline and freshwater influx in some of the species-pair locations, to test the theory that a double peak in relative sea-level (RSL) over the Holocene facilitated a double-invasion of anadromous stickleback, intersected by a period of evolution in allopatry. I found no evidence for such a sequence of saline-freshwater transitions in any of the lochs on North Uist, implying that these species-pairs did not evolve as a result of a recent double-invasion (Chapter 3). I went on to show that North Uist is a meeting place for anciently diverged and predominantly geographically isolated trans-Atlantic and European lineages. Furthermore, I established that these mitochondrial lineages were highly

differentially distributed across ecotypes, which suggested that the trans-Atlantic lineage has failed to establish in freshwater and resident populations, and that conflicts arising as a result of the secondary contact of these lineages may have contributed to maintaining isolation in species-pairs (Chapter 3).

I performed a large mate choice experiment in which among ecotype differences in mating preferences were compared across sympatric and allopatric populations, to test for reinforcement in species-pairs. This demonstrated that females of all ecotypes exhibited assortative mating based on characteristics specific to ecotypes, but this assortative mating appeared to have evolved as a by-product of other divergence, rather than through reinforcement. Furthermore, I also showed that mating preferences were not driven by body size differences, despite there being a correlation between the body size of resident and anadromous ecotypes across lochs containing species-pairs, which was consistent with character displacement in body size (Chapter 4).

Finally, I took a change of direction, to capitalize on a chance finding during the course of my PhD, and investigated the mechanism by which a stickleback, a normally oviparous species, came to be carrying well-developed embryos within the ovaries. I showed that the embryos could not have been produced by hermaphroditism, or parthenogenesis, and thus must have been fertilised by the entry of sperm from a separate, male individual into the reproductive tract. I also investigated the prevalence of prolonged egg-retention in the population from which the female carrying well-developed embryos was found, and showed that a higher proportion of female stickleback become egg-bound during the breeding season in this population than on the rest of North Uist. This suggests that the

tendency to retain eggs for a prolonged period could be a preadaptation for the major life history transition from oviparity to ovoviviparity.

6.2 Ecological vs. genetic speciation

In this thesis, I have focused on speciation in stickleback, but similar processes are likely to be important for speciation in other species. Ecologically based divergent selection has become extremely popular as a mechanism for speciation across many taxa (Nosil, 2012), not least because it can theoretically occur regardless of spatial or geographical context (Schluter, 2001). Overall, this project provided only very limited evidence for an ecological basis to divergence in stickleback. Chapter 2 indicated that the presence of additional intraspecific morphs or ecotypes could itself be involved in constraining speciation. This suggests that biotic ecological interactions may be particularly important during speciation. However, while it highlights the role of ecological differences between environments in constraining speciation, it does not determine whether speciation, in the absence of additional ecotypes, had an ecological basis. Furthermore, ideally, multiple replicates of tri-ecotype contact zones would have been utilised to characterise the pattern of divergence in Uist stickleback populations, and to give a more concrete basis for the conclusions in Chapter 2. These contact zones are rare in stickleback (Campbell, 1985), and so this was not possible during this investigation, but further work to identify and characterise RI across similar contact zones in other species would be particularly interesting, and would add weight to the theories developed in Chapter 2.

There was also some suggestion of signatures of character displacement in body size in Chapter 4, which could well have an ecological basis, and has been identified and used as evidence for ecological speciation in other stickleback populations (Nagel and Schluter, 1998; Schluter and McPhail, 1992), as well as other taxa (Fenchel, 1975; Giannasi *et al.*, 2000). However, given that anadromous fish only spend a very small proportion of their lives in the lochs this seems somewhat unlikely in this case. Mate choice experiments in Chapter 4 also indicated that male stickleback of different ecotypes differed in terms of the substrate on which they preferred to build their nests, indicating that there could be spatial segregation between ecotypes during spawning. This implies a role for ecological pre-mating isolation in species-pairs, as is the case in other sympatrically spawning fish species (Nagelkerke and Sibbing, 1996; Zimmerman and Reeves, 2000). Further research into these factors, which may indicate an ecological basis for RI, is, therefore, merited.

Ecologically based RI can develop rapidly (Hendry *et al.*, 2007), whereas it generally takes a long period of time for neutral genetic differences to accumulate. Thus, if, in Chapter 3, we had found evidence that a recent double-invasion was responsible for speciation in stickleback species-pairs, this would have been more consistent with RI having developed as a result of purely ecological factors. However, our results indicated that RI in species-pairs is more likely to be underlain by incompatibilities between two anciently diverged mitochondrial lineages, which appear to be experiencing recent secondary contact, than a recent Holocene double-invasion. These mitochondrial lineages diverged approximately during the Eemian interglacial period ~115,000 – 130,000 YBP (Chapter 3, Makinen and Merila, 2008), and so genetic

incompatibilities would have had ample time to accumulate, and are likely to also be involved in maintaining RI. Indeed we identified 17 non-synonymous (protein altering) changes in the cyt *b* gene across trans-Atlantic and European lineages, only one of which was shared between the two lineages (Chapter 3). A particularly interesting extension to this project would be to investigate these changes further, and identify potential consequences for the structure and function of the Cyt b protein, and its role in the electron transport chain.

The indication that genetic incompatibilities may be important does not, of course, rule out a role for ecological factors as well, particularly given the two lineages were probably maintained in refugia on opposite sides of the Atlantic, and therefore almost certainly also experienced divergent ecological conditions during their allopatric phase. This research, therefore, indicates that both ecological and genetic factors have probably been important in shaping patterns of diversification and reproductive isolation in stickleback species-pairs on North Uist, and more detailed investigations to identify the specifics of these factors is warranted. This outcome is consistent with Hendry (2009)'s verdict that speciation in stickleback does not progress further than weak to modest RI when based on ecological factors alone, and is also concordant with the recent suggestion that seemingly recent, purely ecological speciation may in fact often be underlain by much older genetic divergence (Feder *et al.*, 2005).

6.3 The importance of secondary contact in speciation

Major vicariant events, including geological and geomorphological changes, capable of drastically altering the connectivity of different environments, are occurring almost constantly (within the time frame of evolution). For example,

processes such as erosion, landslides, flooding and RSL change (all of which may be related to glacial processes) can rapidly modify the structure of entire freshwater and coastal habitats. These environments are also particularly vulnerable to connectivity changes because of their naturally fragmented nature, and the fact that rivers, streams and entire catchments can be abruptly shifted to drain elsewhere (Hooke, 2007; Korup, 2004; Wells and Dorr, 1987). The ecological effects of these changes for population distributions are rarely considered in relation to speciation (Chesser and Zink, 1994; Doebeli and Dieckmann, 2003; Foote, 2018; Grant and Grant, 2009; Moura *et al.*, 2015), but have massive implications for debates regarding the importance of spatial isolation, secondary contact and speciation by reinforcement.

We investigated two possible scenarios in which secondary contact would have been important for RI in species-pairs. We showed that a recent (Holocene) double invasion is unlikely to have contributed to RI (Chapter 3), and mate choice experiments were consistent with this, providing no indication of reinforcement in species-pairs (Chapter 4). Although we did not find evidence for a recent double-invasion, the multidisciplinary approach utilised in Chapter 3 to test this hypothesis allowed us to gain insights into the geographic history of stickleback populations on North Uist, that would have been impossible to achieve by other means, and it highlights the importance of combining the study of both palaeo and modern material. As technology, and our ability to extract information from ancient material improves, this is likely to become an extremely exciting and fruitful area of research, and the contribution of other palaeo data, such as ancient DNA will add further merit to reconstructions such

as these, and could significantly impact our understanding of how spatial isolation has influenced speciation.

Further investigation revealed that, in all probability, secondary contact between anciently diverged mitochondrial lineages was involved in maintaining isolation, indicating that secondary contact probably was important for the evolution of species-pairs, as is the case in many other taxa (Jowers *et al.*, 2014; Martin-Bravo *et al.*, 2010; Quenouille *et al.*, 2011; Schield *et al.*, 2017). This hypothesis is also more consistent with the broad scale distribution of isolation between ecotypes in stickleback, as if RSL change was responsible for a double invasion on North Uist it would have been likely to have had a similar effect elsewhere, as the Pleistocene glaciers retreated across northern Europe. Yet, the occurrence of resident and anadromous ecotypes, in the absence of intermediate morphs, is extremely rare, occurring in only a handful of other isolated locations (Drevecky *et al.*, 2013; Karve *et al.*, 2008; Ziuganov, 1995). The trans-Atlantic and European mitochondrial lineages, on the other hand, occupy mostly geographically discrete locations, with contact predominantly restricted to the west coast of Scotland and Ireland (Makinen and Merila, 2008; Ravinet *et al.*, 2014a), although this might be expanded by more extensive sampling across Europe.

The Pleistocene glaciers were responsible for restricting the ranges of many other species to multiple isolated refugia, during which time substantial divergence often occurred (Hewitt, 2000; Ohtani *et al.*, 2013; Pinceel *et al.*, 2005; Quesada *et al.*, 1995; Rohfritsch and Borsa, 2005; Vila *et al.*, 2005). Signatures of this divergence are also still present in many other species,

particularly in mtDNA (Lovette, 2005), presumably because of its general tendency to be non-recombinant, and in some cases epistasis between mitochondrial and nuclear DNA is now responsible for RI (Dasmahapatra *et al.*, 2002). The idea that mtDNA can be under selection, and potentially play a role in speciation is a relatively new and exciting area of research (Burton and Barreto, 2012; Burton *et al.*, 2013; Hill, 2016; 2017; 2018; Sloan *et al.*, 2017), and has only very recently begun to be considered as a mechanism for speciation in stickleback (Lescak *et al.*, 2017). Nevertheless, the marine-freshwater radiation in stickleback provides an ideal system in which to study mitonuclear conflicts and speciation because transitioning from a marine to a fresh or brackish existence entails major physiological changes that mitochondrially encoded proteins are important for (Jacoby *et al.*, 2011; Paital and Chainy, 2012; Sakamoto *et al.*, 2001). In addition, it appears that stickleback mitochondrial lineages are often differentially successful in making the transition from salt to freshwater, as we have identified this characteristic on North Uist (Chapter 3), and it has been previously recorded in other locations (Ravinet *et al.*, 2014b). It is, therefore, highly plausible that mitonuclear conflict may be involved in maintaining RI across multiple marine-freshwater transitions in stickleback.

This investigation, therefore, adds to the growing body of literature which suggests that seemingly sympatric speciation may often reflect secondary contact, with prior differentiation in allopatry (Bernatchez and Dodson, 1990; Feder *et al.*, 2003; Foote and Morin, 2015; Kuehne *et al.*, 2007). Nonetheless, the findings of this study alone, do not determine a causal relationship between secondary contact between these ancient lineages and speciation conclusively, but rather, they open the door for further investigations into possible epistatic

interactions between mitochondrial and nuclear DNA in populations where these lineages meet, and into the mitochondrial background of locations on the island where hybridisation does occur, such as the tri-ecotype contact zone identified in Chapter 2. Future work could include in vitro assays of mitochondrial function across extant ecotypes and lineages, and in forced in vitro crosses between apparently incompatible ecotype-lineage combinations. It could also include codon-based analyses of selection on mitochondrial genomes and accompanying autosomal regions in different lineages, to identify whether any of the non-synonymous changes identified here, and any related autosomal changes, exhibit signatures of positive selection.

6.4 Asymmetry in reproductive isolation

Asymmetric reproductive isolation is surprisingly common (Arnold *et al.*, 1996; Panhuis *et al.*, 2001; Tiffin *et al.*, 2001; Wu *et al.*, 1995), particularly when mitonuclear interactions are involved, because the mitochondria is almost always maternally inherited, causing potential conflict between the sexes (Ellison and Burton, 2008). In Chapter 3, I highlighted the fact that asymmetric mate choice, with small resident females being prepared to choose large anadromous males, but large anadromous females avoiding small resident males, could play a role in preventing the trans-Atlantic mitochondrial lineage from entering resident stickleback populations. Mate choice experiments in Chapter 4 supported this hypothesis as no trials involving anadromous females crossed with resident males never resulted in spawning (although only a relatively small number of trials with this pairing could be attempted due to a lack of resident male nests), yet spawning occurred in 10% of trials involving resident females

and anadromous males. This, coupled with the fact that asymmetrical mating preferences are known to play a vital role in maintaining RI in other stickleback species-pairs (Kitano *et al.*, 2007), strongly implies that they could be important for North Uist pairs. In Chapter 3, I also theorised that this asymmetry in female mate choice could be driven by body size, but mate choice experiments in Chapter 4 suggested that female preference was not influenced by body size. This suggests that either the effect of body size on mating preferences was too small to detect in our experiments (but this seems unlikely given that it was a relatively large experiment), or mate choice was based on characteristics of the individual ecotypes that were not quantified in our experiments. Further experiments would therefore be extremely useful to shed light on questions which remain unanswered in Chapter 4, particularly whether anadromous females consistently possess a total aversion to mating with resident males, and which other characteristics, possessed by resident and anadromous ecotypes, are responsible for assortative mating.

6.5 Concluding remarks

I have shown that the stickleback populations on North Uist are particularly interesting from a speciation perspective. They encompass an exciting array of both phenotypic divergence and genetic isolation, alongside extensive hybridisation, secondary contact between ancient lineages and even population differences in reproductive biology which could predispose them to evolving major life history transitions. Studying these populations has highlighted the potential influence of intraspecific ecological interactions between ecotypes on speciation, shed light on the contribution of recent and ancient geographic events

to shaping population distributions and divergence, and given us new insights into the evolutionary mechanisms that underlie RI. Furthermore, it has allowed us to witness and gain insights into an exciting and anomalous incidence of natural history. Arguably, however, the most important thing to have come from this research is an exciting new array of questions, which merit further investigation and provide direction to future research.

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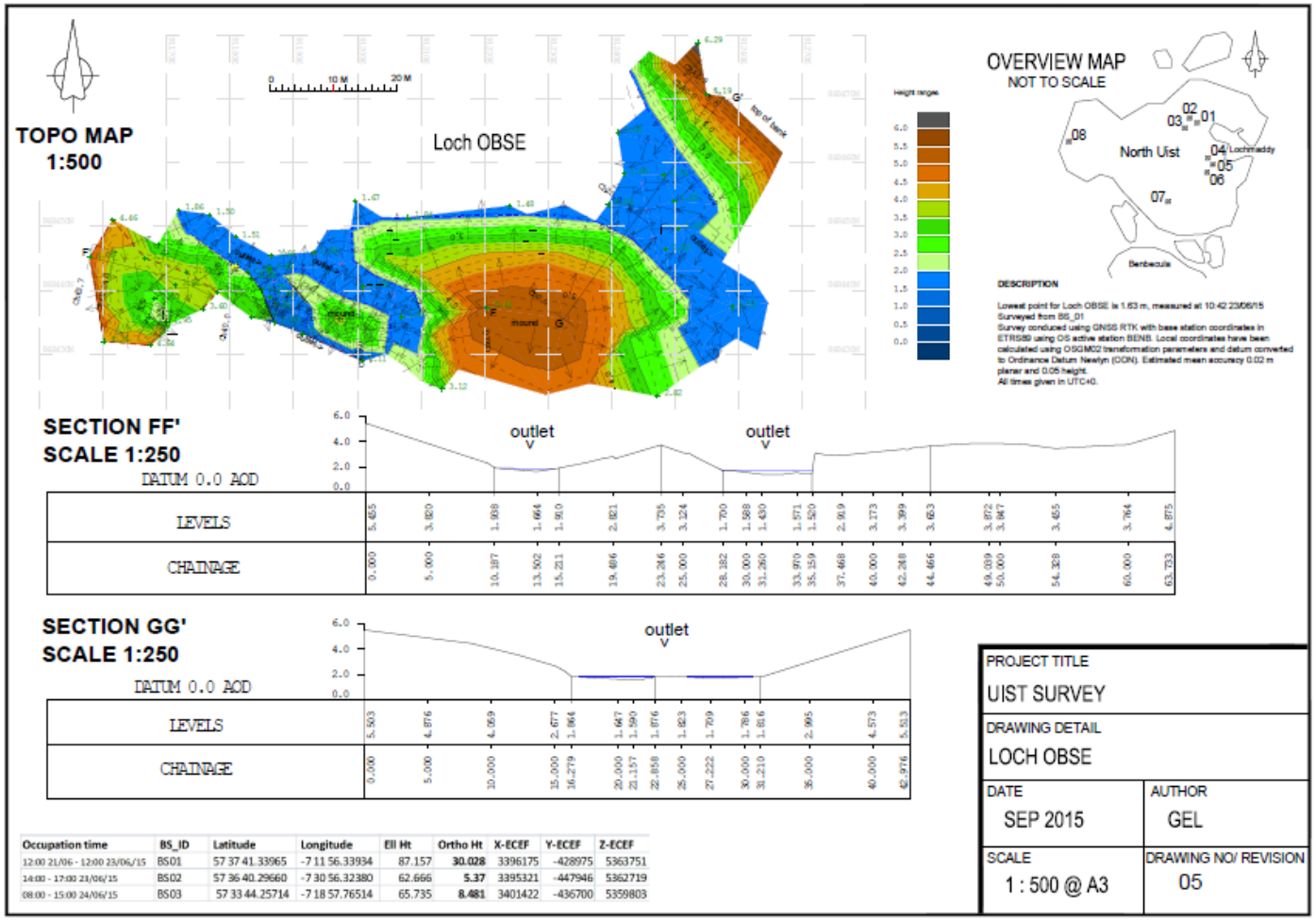
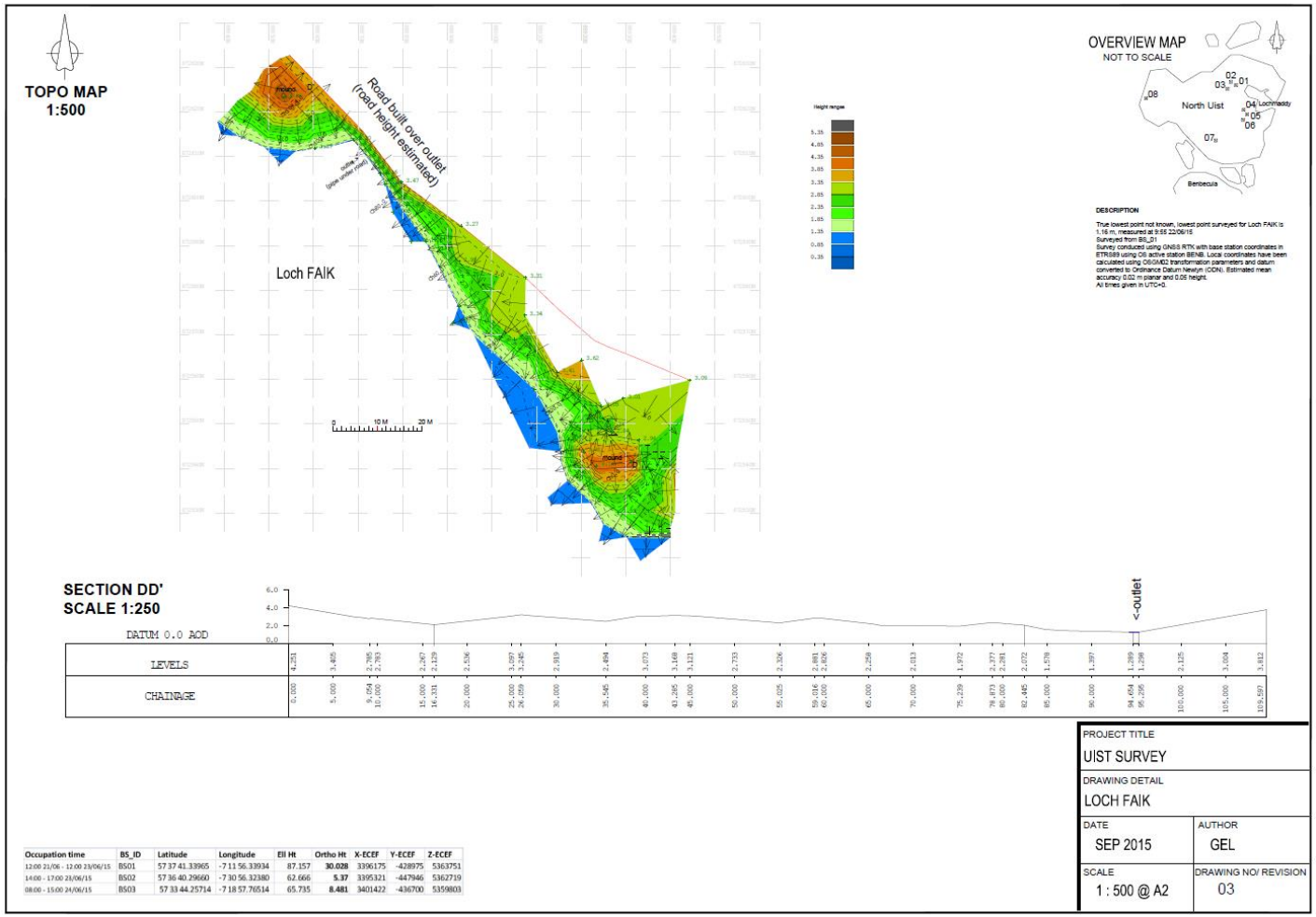
SUPPLEMENTARY MATERIAL

Figure S1 Depth – age correlations for Faik. Calibrated radiocarbon dates (grey circles) with the standard deviation indicated by associated error bars. The lm fit is indicated by the solid black line.



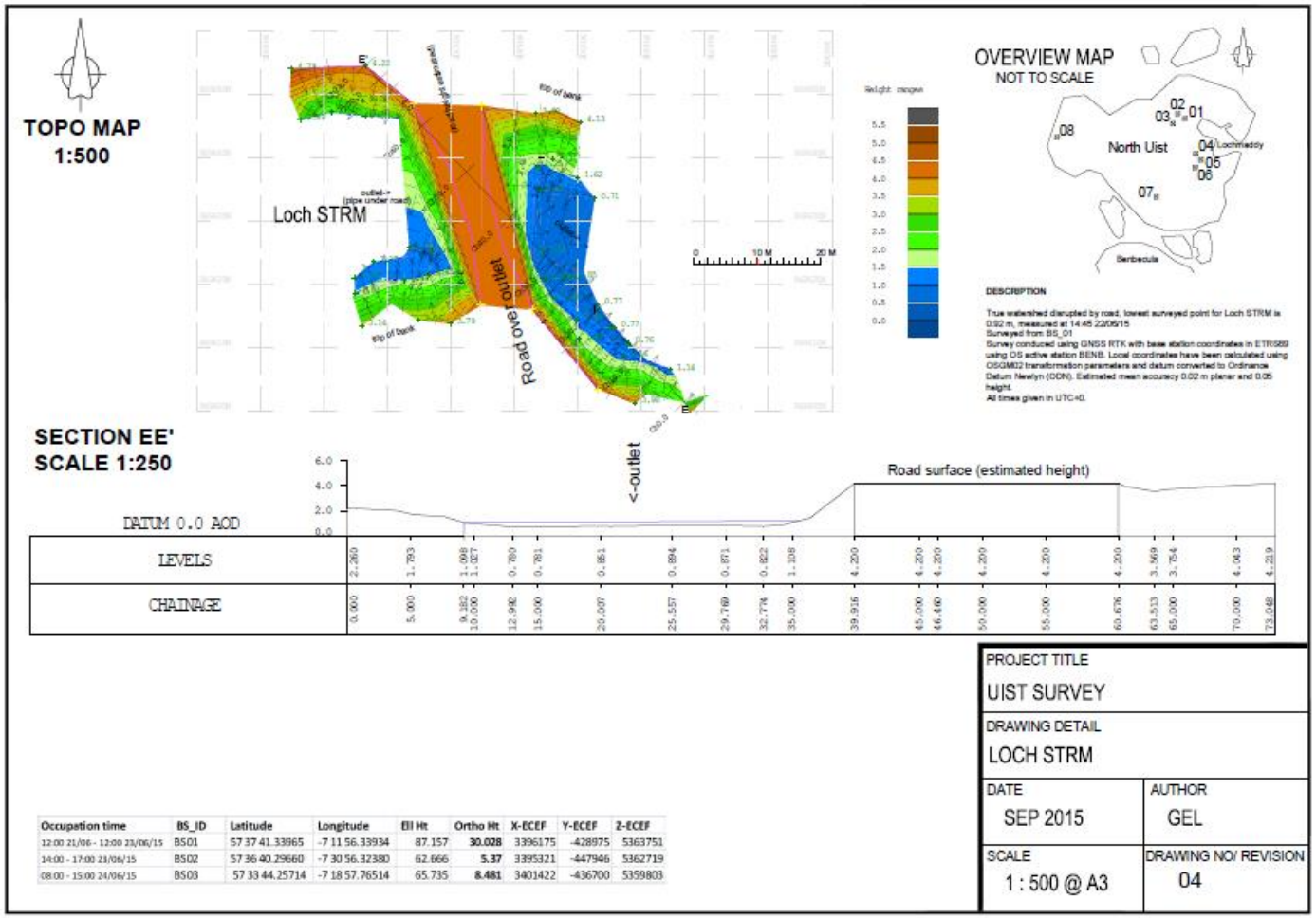
Occupation time	BS_ID	Latitude	Longitude	Ell Ht	Ortho Ht	X-ECEF	Y-ECEF	Z-ECEF
12:00 21/06 - 12:00 23/06/15	BS01	57 37 41.33965	-7 11 56.33934	87.157	**30.028**	3396175	-428975	5363751
14:00 - 17:00 23/06/15	BS02	57 36 40.29660	-7 30 56.32380	62.666	**5.37**	3395321	-447946	5362719
08:00 - 15:00 24/06/15	BS03	57 33 44.25714	-7 18 57.76514	65.735	**8.481**	3401422	-436700	5359803

Figure S2 Topographical digital terrain map of Obse outlet.



Occupation time	BS_ID	Latitude	Longitude	Ell Ht	Ortho Ht	X-ECEF	Y-ECEF	Z-ECEF
12:00 21/06 - 12:00 23/06/15	BS01	57 37 41.33965	-7 11 56.33934	87.157	**30.028**	3396175	-428975	5363751
14:00 - 17:00 21/06/15	BS02	57 36 40.29660	-7 30 56.32380	62.666	**5.37**	3395321	-447946	5362719
08:00 - 15:00 24/06/15	BS03	57 33 44.25714	-7 18 57.76514	65.735	**8.481**	3401422	-436700	5359803

Figure S3 Topographical digital terrain map of Faik outlet.



Occupation time	BS_ID	Latitude	Longitude	Ell Ht	Ortho Ht	X-ECEF	Y-ECEF	Z-ECEF
12:00 21/06 - 12:00 23/06/15	BS01	57 37 41.33865	-7 11 56.33934	87.157	**30.028**	3396175	-428975	5363751
14:00 - 17:00 23/06/15	BS02	57 36 40.29660	-7 30 56.32380	62.666	**5.37**	3395321	-447946	5362719
08:00 - 15:00 24/06/15	BS03	57 33 44.25714	-7 18 57.76514	65.735	**8.481**	3401422	-436700	5359803

Figure S4 Topographical digital terrain map of Strm outlet.

Table S1 Diatom species identified in Obse, North Uist. Classifications follow Van Dam *et al.* (1994).

Genus	Species	Total number of valves identified
Actinocyclus	*normanii*	1
Achnanthes	*conspicua*	2
Achnanthes	*daonensis*	2
Achnanthes	*flexella*	3
Achnanthes	*laevis*	2
Achnanthes	*linearis*	9
Achnanthes	*minutissima*	193
Achnanthes	*oblongella*	6
Achnanthes	*pseudoswazi*	4
Achnanthes	*pusilla*	1
Achnanthes	Spp.	5
Achnanthes	*subatomoides*	3
Amphora	*coffeaeformis*	2
Amphora	*libyca*	1
Amphora	*montata*	4
Amphora	Spp.	2
Amphora	*veneta*	10
Anomoeoneis	*brachysira*	82
Anomoeoneis	*vitrea*	376
Aulacoseira	*alpigena*	2
Aulacoseira	Spp.	2
Caloneis	*undulata*	1
Cocconeis	*disculus*	1
Cocconeis	*neodiminuta*	23
Cocconeis	*placentula*	13
Cocconeis	*scutellum*	20
Cocconeis	Spp.	5
Cocconeis	Spp.	1
Cyclotella	*antiqua*	4
Cyclotella	*atomus*	18
Cyclotella	*bodanica*	4
Cyclotella	*clyclopuncta*	6
Cyclotella	*distinguenda*	18
Cyclotella	*ocellata*	37

Genus	Species	Total number of valves identified
Cyclotella	*stelligera*	2
Cyclotella	*striata*	1
Cymbella	*affinis*	3
Cymbella	*cesatii*	61
Cymbella	*cistula*	1
Cymbella	*gaeumannii*	5
Cymbella	*gracilis*	57
Cymbella	*incerta*	5
Cymbella	*microcephala*	4
Cymbella	*microcephala-like*	27
Cymbella	*perpusilla*	3
Cymbella	*silesiaca*	1
Cymbella	Spp.	3
Denticula	*kuetzingii*	1
Diploneis	*finnica*	1
Diploneis	*interrupta*	4
Diploneis	*modica*	1
Epithemia	*sorex*	20
Epithemia	Spp.	3
Eunotia	*arcus*	7
Eunotia	*bilunaris var. mucophila*	22
Eunotia	*elegans*	3
Eunotia	*faba*	4
Eunotia	*incisa*	11
Eunotia	*intermedia*	5
Eunotia	*monodon*	1
Eunotia	*muscicola*	1
Eunotia	Spp.	10
Eunotia	*sterineckii*	7
Fragilaria	*brevistricta*	8
Fragilaria	*capucina*	2
Fragilaria	*construens*	9
Fragilaria	*exigua*	319
Fragilaria	*pseudoconstruens*	17

Genus	Species	Total number of valves identified
Fragilaria	Spp.	3
Frustulia	*rhomboides*	144
Gomphonema	*acuminatum*	9
Gomphonema	*gracile*	3
Gomphonema	Spp.	3
Navicula	*angusta*	28
Navicula	*begerii*	3
Navicula	*cryptocephala*	3
Navicula	*cryptotenella*	60
Navicula	*digitulus*	1
Navicula	*forcipata*	2
Navicula	*halophila*	1
Navicula	*ignota*	1
Navicula	*krasskei*	1
Navicula	*mediocris*	6
Navicula	*pseudoarvensis*	3
Navicula	*pseudoscutiformis*	1
Navicula	*pupula*	4
Navicula	*pygmaea*	1
Navicula	*salinarum*	1
Navicula	*seminuscula*	2
Navicula	*soehrensis*	7
Navicula	Spp.	11
Navicula	*subtilissima*	20
Neidium	*affine*	1
Neidium	*alpinum*	1
Neidium	*septentrionale*	2
Neidium	Spp.	1
Nitzshica	*angustata*	2
Nitzschia	*frustulum*	3
Nitzschia	*hungarica*	1
Nitzschia	*nana*	3
Nitzschia	*palea*	16
Nitzschia	*perminuta*	1

Genus	Species	Total number of valves identified
Nitzschia	Spp.	26
Pinnularia	*gibba*	3
Pinnularia	*interrupta*	6
Pinnularia	*microstauron*	2
Pinnularia	*viridis*	2
Stauroneis	*anceps*	1
Stauroneis	*phoenicenteron*	3
Surirella	*amphioxys*	1
Rhopalodia	*gibba*	3
Rhopalodia	*rupestris*	7
Tabellaria	*flocculosa*	38
Tetracyclus	*rupestris*	1
Unknown	unknown	5