THE GENETICS OF ADAPTATION IN STICKLEBACK

by

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Abstract

Understanding the process of adaptation requires elucidating the mechanisms through which natural selection alters the genetic variation underlying phenotypic traits. Here, I explore the genetics of adaptive evolution empirically, using lab and field experiments with threespine stickleback fish, and theoretically, using population genetic models. Freshwater stickleback populations are derived from ancestral marine populations that colonized lakes and streams at the end of the last ice age. These derived populations exhibit remarkable parallel divergence in a number of morphological and physiological traits. The parallel nature of these changes suggests the influence of natural selection, because genetic drift is unlikely to produce a strong correlation between phenotype and environment. Adaptive evolution in some of these traits is due to selection on standing genetic variation present in the ancestral marine population. I investigate the ecological, ontogenetic and behavioural mechanisms that contribute to the maintenance of this variation. I present evidence of extremely strong selection acting at phenotypic and genotypic levels over short time scales. Population genetic theory typically assumes much smaller selection coefficients than those measured in this work. I derive population genetic theory to describe the distribution of fitness effects of beneficial mutations without this restriction of weak selection, and test the analytical theory with numerical simulations. Collectively, this research is helping to identify some of the primary functional mechanisms that maintain genetic variation within and between natural populations.

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Co-authorship statement

Chapter 2: Rowan Barrett conceived the study, Rowan Barrett wrote the manuscript with input from Dolph Schluter.

Chapter 3: Rowan Barrett, Sean Rogers and Dolph Schluter designed the study, Rowan Barrett and Sean Rogers performed experiments, Rowan Barrett and Dolph Schluter analyzed the data, Rowan Barrett wrote the manuscript with input from Sean Rogers and Dolph Schluter.

Chapter 4: Rowan Barrett, Sean Rogers and Dolph Schluter designed the study, Rowan Barrett and Sean Rogers performed experiments, Rowan Barrett and Dolph Schluter analyzed the data, Rowan Barrett wrote the manuscript with input from Sean Rogers and Dolph Schluter.

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Chapter 8: Rowan Barrett, Leithen M'Gonigle and Sarah Otto designed the study, Leithen M'Gonigle and Sarah Otto developed numerical simulations and analytical theory, Rowan Barrett, Leithen M'Gonigle and Sarah Otto analyzed the data,

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1. INTRODUCTION

With over 100 million species and a spectacular array of ecological roles, the biological world is incredibly diverse. Understanding the processes responsible for biodiversity is one of the most fundamental challenges to human inquiry. How new species arise, what Charles Darwin referred to as the "mystery of mysteries", remains one of the most important questions in biology. One of Darwin's most radical ideas was that new species originate by the process of divergent natural selection. The process of adapting to unique selection pressures in different environments provides the engine that drives the continual production of biological diversity. Understanding how organisms adapt is thus essential for addressing Darwin's mystery of mysteries. This line of inquiry is also profoundly important for understanding the wide-ranging effects that humans are having on the evolutionary trajectory of life on this planet.

The recent invention of rapid genome sequencing now allows the molecular dissection of adaptation in ways that Darwin could never have imagined. Paradoxically, the trend towards reductionism in much of biology makes it possible to take a more holistic and integrated approach in evolutionary biology in which the integration of molecular, developmental and ecological information provides novel insight to key questions. My thesis aims to synthesize knowledge from different levels of biological organization to better understand the mechanisms that drive biological diversification. By investigating the genetic basis and ecological processes responsible for evolutionary change in nature, the work described here helps to reveal emergent properties of interactions between genes and the environment. As we will see, these higher-level natural phenomena can alter selective outcomes in ways that are not predictable from

laboratory studies.

The following chapters are independent manuscripts containing more detailed background than will be covered in this brief introductory chapter. Because all chapters have already been published, there is some redundancy throughout the thesis. This redundancy does not concern the topics covered, but rather many of the references used, some components of the figures, and description of the stickleback system. I hope that the summary of chapters below will help readers keep track of how each chapter fits into the overall larger picture.

Chapter 2 provides a review of adaptation from standing genetic variation. Populations adapt to novel environments in two distinct ways: selection on pre-existing genetic variation and selection on new mutations. These alternate sources of beneficial alleles can result in different evolutionary dynamics and distinct genetic outcomes. Compared with new mutations, adaptation from standing genetic variation is likely to lead to faster evolution, the fixation of more alleles of small effect and the spread of more recessive alleles (Orr and Betancourt 2001; Hermisson and Pennings 2005; Przeworski et al. 2005; Pennings and Hermisson 2006a, b). There is potential to distinguish adaptation from standing variation by differences in the genomic signature of selection (Przeworski et al. 2005; Teshima et al. 2006). I review these approaches and possible examples of adaptation from standing variation in natural populations. Understanding how the source of genetic variation affects adaptation will be integral for predicting how populations will respond to changing environments.

The bulk of the thesis (Chapters 3-6) investigates the genetics of adaptation in the fish threespine stickleback (*Gasterosteus aculeatus*) by testing functional mechanisms responsible for the maintenance of standing variation at a major effect gene. Threespine

sticklebacks are a leading example of an ecological model system with well-developed genomic tools (Kingsley et al. 2004; Gibson 2005; Peichel 2005). The stickleback has unique advantages for studying the genetics of adaptation, namely because they are among the youngest species on Earth and their recent bouts of evolution in newly colonized post-glacial lakes represent natural laboratories within which the evolutionary process can be studied in the wild (Hagen and McPhail 1970; Bell and Foster 1994; McPhail 1994; Taylor and McPhail 1999). Derived freshwater stickleback populations have undergone a number of repeated morphological changes from their marine ancestors, including changes to body size, body shape, pigmentation, and bony defensive armour (McKinnon and Rundle 2002). Candidate genes responsible for several of these phenotypic differences have recently been identified (Colosimo et al. 2004; Shapiro et al. 2004; Colosimo et al. 2005; Miller et al. 2007). Chapters 3-6 focus on the major effect gene *Ectodysplasin* (*Eda*), which controls variation in lateral plate armour (Colosimo et al. 2005). A derived allele (low) causing reduced plate number has been fixed repeatedly after marine stickleback colonized freshwater from the sea, where the ancestral allele (complete) predominates.

In Chapter 3, I describe a transplant experiment with selected genetic variants to evaluate the fitness consequences arising from the functional effects of *Eda* in nature. I introduced wild marine sticklebacks carrying one low allele and one complete allele to replicated freshwater environments. The low allele increased in frequency once lateral plates developed, most likely via a growth advantage. Opposing selection at the juvenile stage and changing dominance for fitness throughout life suggest either that the gene affects additional traits undergoing selection or that linked loci are also affecting fitness.

The study demonstrates the utility of direct measurements of selection on genes underlying traits for elucidating the mechanisms of evolution.

Chapter 4 represents a more detailed examination of the functional effects of *Eda* under controlled laboratory settings where abiotic parameters could be manipulated to test for environment specific pleiotropic effects. When raised in freshwater, reduced armor sticklebacks carrying low alleles at *Eda* had increased growth rate relative to fully armored sticklebacks carrying complete alleles. In saltwater treatments this growth advantage was present during juvenile growth but lost during adult growth, suggesting that in this environment stickleback are able to develop full armor plates without sacrificing overall growth rate. The environment-specific pleiotropic effects of *Eda* demonstrate that ecological factors can mediate the influence of genetic architecture in driving phenotypic evolution.

In Chapter 5, I continue to investigate potential mechanisms allowing the maintenance of variation at *Eda* by investigating if the alleles conferring local adaptation are associated with behavioural differences. Adaptive divergence in armour between marine and freshwater populations would be facilitated if the low allele conferred a behavioural preference for freshwater environments. I experimentally tested whether the low allele is associated with preference for freshwater by measuring the preference of each *Eda* genotype for freshwater versus saltwater after acclimation to either salinity level. I found no association between the *Eda* low allele and preference for freshwater. Instead, the low allele was significantly associated with a reduced preference for the acclimation environment. This behaviour may facilitate the colonization of freshwater

habitats from the sea, but it could also hinder local adaptation by promoting migration of low alleles between marine and freshwater environments.

Chapter 6 is a review that synthesizes recent work on *Eda* with the extensive body of research on lateral plate phenotypes. The aim is to provide a case study that demonstrates the utility of starting with a phenotype of interest, identifying genotype, and finally evaluating the fitness consequences arising from the phenotypic effects of specific alleles under natural conditions. This approach can improve the chances that underlying phenotypes and genotypes are relevant for adaptation, thus improving our understanding of the ecological mechanisms responsible for evolutionary change in natural populations.

In Chapter 7, I investigate the evolution of temperature tolerance in freshwater stickleback populations. Physiological differences between ancestral marine and derived freshwater populations have received far less research attention than morphological traits, but they undoubtedly play an important role in adaptation to freshwater environments. I show that freshwater sticklebacks are able to tolerate lower minimum temperatures than marine sticklebacks and that this difference is heritable. I transplanted marine sticklebacks to freshwater ponds and measured the rate of evolution after three generations in this environment. Cold tolerance evolved at a rate of 0.63 *haldanes* to 2.5 °C lower than the ancestral population, matching the cold tolerance found in wild freshwater populations. These results suggest that cold tolerance is under strong selection and that marine stickleback carry sufficient genetic variation to adapt to changes in temperature over remarkably short time scales.

Chapter 8 is a theoretical study investigating the distribution of fitness effects of beneficial mutations. Understanding the characteristics of this distribution is essential for

the development of a general theory of adaptation. Theoretical derivations of the distribution have assumed small selection coefficients, despite strong selection being observed in some experiments, especially those involving novel environments. For example, the selection coefficients observed in my transplant experiments with stickleback are an order of magnitude larger than those assumed in most theory. I derive the distribution of fitness effects among fixed beneficial mutants without the restriction of low selection coefficients. The fate of strongly favoured alleles is less affected by stochastic drift while rare, causing the distribution of fitness effects among fixed beneficial mutations to reflect more closely the distribution among all newly arising beneficial mutations.

In the final chapter of the thesis I draw some general conclusions from my research and outline a few unresolved issues concerning the genetics of adaptation, in sticklebacks specifically and among taxa more generally. I also suggest some potential avenues for future research into this topic.

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2. ADAPTATION FROM STANDING GENETIC VARIATION[1]

Introduction

When a population colonizes a new environment or experiences a novel selective pressure, does it adapt mainly from standing genetic variation or does it wait for new mutations? There are good reasons to ask this question. First, adaptation is likely to be faster from standing variation than from new mutation, not only because beneficial alleles are immediately available, but also because they usually start at higher frequencies (Innan and Kim 2004). As humans alter the biosphere, forcing many species to confront dramatically altered environments; it is becoming increasingly important to understand how rapidly populations can adapt (Palumbi 2001; Franks et al. 2007; Bradshaw and McNeilly 1991).

Another reason is that a beneficial allele present as standing variation is older than a new mutation, and might have been pre-tested by selection in past environments, in another part of the species' range, or even in another species with which the population has exchanged genes (Rieseberg et al. 2003). Such alleles might have multiple advantageous genetic changes (Liti et al. 2006; McGregor et al. 2007). In contrast to new mutations, such standing variation has already passed through a "selective filter", which increases the chance that large-effect alleles are advantageous, and the probability of parallel evolution (Schluter et al. 2004).

A third reason is that the molecular signature of selection, which is often the only evidence available that a gene has recently fixed under directional selection, is not the

[1] A version of this chapter has been published. Barrett, R.D.H. and Schluter, D. 2008. Adaptation from standing genetic variation. *Trends in Ecology and Evolution* 23(1): 38-44.

same when a population adapts from standing variation instead of from new mutations (Przeworski et al. 2005). Finally, understanding the source of variation for adaptation might tell us a great deal about the factors maintaining genetic variation in natural populations, still one of the most debated topics in evolution (Hedrick 1986; Hedrick 2006; Lynch and Walsh 1998; Barton and Keightley 2002).

Of course, we already have part of the answer. A century of quantitative genetics has established the ubiquity of standing variation in natural populations, which successfully predicts the short-term response to selection (Hill 1982; Roff 2007). At the other extreme, macroevolution would surely not be possible without a steady supply of new mutations over the long term. Yet these facts do not completely establish the relative roles of standing variation and new mutation during adaptation to an altered environment. The surest way to determine the source of beneficial alleles is to locate the genes themselves and establish their histories. Here we review the consequences of adaptation from standing genetic variation, contrast it with adaptation from new mutations, and identify ways in which it is possible to tell the difference.

How the source of beneficial alleles affects the genetics of adaptation

The process of adaptation from standing genetic variation is expected to differ in several ways from adaptation based on new mutations. We summarize several of these differences here. Our summary is not exhaustive, but then neither is the literature. Despite overwhelming observational and experimental evidence for the role of natural selection in phenotypic evolution, theoretical investigation of the selective effects of alleles contributing to adaptation is relatively new (reviewed in Orr 2005a; Orr 2005b). Most

current theory on the genetics of adaptation assumes that adaptation occurs exclusively from new mutations rather than from standing variation. The theory for standing variation we summarize below assumes that newly beneficial alleles are neutral or deleterious prior to the change of environment and are maintained in the ancestral population through a balance of recurrent mutation, selection and drift (Przeworski et al. 2005; Hermisson and Pennings 2005; Orr and Betancourt 2001).

Probability of fixation

All else being equal, the chance that an advantageous allele becomes fixed in a population, rather than lost by genetic drift, is greater if it is present in multiple copies (standing variation) than if it appears as a single new mutation (Figure 2.1). The probability of fixation increases with the magnitude of the beneficial effect (s_b) and with increasing effective population size (N_e) in both scenarios; however, over a large range of selective effects, the probability of fixation is high for standing variation when it is negligible for a new mutation (Hermisson and Pennings 2005). This increase in fixation probability from standing variation is especially great for small effect mutations, suggesting that small effect alleles should contribute more to adaptation from standing variation than from new mutation. The exact form of the curve in Figure 2.1 assumes that standing variation was previously neutral, but a greater probability of fixation from standing variation should be general.

Speed of adaptation

Standing variation also leads to more rapid evolution in novel environments because it is

available immediately at the time that selective conditions change, whereas waiting time is needed for a new beneficial mutation to arise. Furthermore, the initially higher frequency of beneficial alleles present as standing variation reduces the average fixation time (Hermisson and Pennings 2005). Simulations of the process of fixation from standing variation nevertheless suggest that in the time it takes for an allele to fix from standing variation the allele will also arise by mutation, assuming that the mutation rate stays high before and after the environmental change (Hermisson and Pennings 2005). Even in this case, most copies of the fixed allele are supplied from standing variation (Hermisson and Pennings 2005). Consequently, alleles from standing variation should dominate in most cases when adaptation occurs over short timescales.

Dominance

There is a strong fixation bias against recessive mutations when adaptation occurs from new mutations because they experience weak selection when rare, a process known as Haldane's sieve (Charlesworth 1992; Turner 1981; Haldane 1927). However, the effect vanishes when adaptation occurs from standing variation (Hermisson and Pennings 2005; Orr and Betancourt 2001). This happens because, although a particular copy of a more dominant advantageous allele will carry a greater chance of fixation, on average there will have been fewer copies present at mutation-selection balance before the environment changed. Assuming that there is a correlation between the size of the deleterious effect before the environmental change and the size of the beneficial effect after the change, these tendencies roughly cancel and, consequently, dominance has little effect on the probability of fixation for advantageous standing variation.

Mechanisms preserving standing variation

The previous section demonstrated that standing variation has a considerable advantage in the speed and probability of fixation. This advantage comes from an assumption that recurrent mutation and drift can maintain these neutral or deleterious alleles at a frequency higher than $^1/_{2N}$ in the ancestral environment. However, there are other factors that could also increase the frequency of alleles present as standing variation above the values predicted from these models. Gene flow from populations experiencing different environmental conditions, or even hybridization with other species, could preserve relatively high amounts of standing variation despite negative selection. Alternatively, alleles that are deleterious under specific environmental or genetic conditions might be hidden from selection because they do not have any effects on phenotype in the ancestral environment. This "cryptic genetic variation" might await an environmental change or introduction of novel alleles before it manifests as a new phenotype (Gibson and Dworkin 2004).

A recent study of oldfield mice by Steiner *et al.* (2007) shows how the genetic background in which alleles are present can mask the effects of ancestral standing variation. In the southeastern USA, *Peromyscus polionotus* has a dark coat, which matches the dark soils of mainland Florida. However, these mice have colonized barrier islands and coastal dunes of Florida's Gulf Coast. These beach mice have a much lighter coat than their mainland conspecifics, presumably a result of selection for camouflage on pale sand dunes (Figure 2.2). The barrier islands are young, <6000 years old, and it is, therefore, likely that the ancestral population is the older mainland subspecies. Two candidate genes, the *melanocortin-1 receptor* (*Mc1r*) and its antagonist, the *Agouti*

signaling protein (*Agouti*), map to independent regions of the genome and together control most of the difference in pigmentation between beach and mainland subspecies (Steiner et al. 2007; Hoekstra et al 2006). Derived alleles (i.e. alleles found in the beach mice) at both loci reduce the level of pigmentation. Moreover, there is a strong epistatic interaction between these two loci: mice homozygous for the dark pigment *Agouti* allele have fully pigmented hairs regardless of their *Mc1r* genotype. This suggests that the *Mc1r* allele producing light pigmentation, presumably deleterious on the darker mainland soil, could be maintained as standing variation in mainland populations, hidden by its epistatic interaction with *Agouti*. As the mice colonized the beach environment, the light pigment *Agouti* allele would be driven to higher frequency by positive selection. In turn, the light pigment *Mc1r* allele would also suddenly become visible to selection. Future population sampling will determine whether the light pigment *Mc1r* allele is present in the ancestral mainland environment (H.E. Hoekstra, personal commun.).

Distinguishing standing variation from new mutations in adaptation

How does one determine whether evolution has used standing variation rather than new mutations? Here, we discuss three approaches that have been used with some success. The first is based on the 'signature of selection', which uses polymorphism data in the genome region linked to a fixed allele to identify a 'selective sweep'. The second involves a demonstration that a fixed allele in a new environment still occurs as standing variation in the ancestral population. The third approach uses a phylogenetic study of the DNA sequences of alternative alleles to determine their origins and their age. None of the approaches is infallible, and we identify possible difficulties with each.

The signature of selection on standing variation

Ever since J.B.S. Haldane's early efforts to determine mutation rates for hemophilia during the 1930s, mathematical models have been used to infer past evolutionary patterns from extant population data (Otto 2000). With the widespread availability of molecular polymorphism data, attention is now focused on identifying patterns in the genome that indicate a recent history of positive selection. The key idea is that the substitution of a beneficial allele at a site in the genome results in "hitchhiking" by neutral alleles at nearby sites physically linked with the selected allele (Kaplan 1989; Maynard Smith and Haigh 1974). The beneficial allele will occur with only a subset of neutral variants at linked sites, creating a nonrandom association or 'linkage disequilibrium' between them. Unless recombination breaks down the association between the selected and neutral sites during the substitution process, a small subset of neutral variants will be fixed along with the selected allele. Thus, the fixation of a beneficial allele will produce a selective sweep that leaves a valley of low polymorphism as a signature in its vicinity in the genome. Although recombination can obscure this signature, potential targets of positive selection can be identified from polymorphism data for recent adaptive fixation events. This approach has been used extensively in *Drosophila* and humans, which both experienced novel selection pressures upon recent expansion out of Africa (e.g. Akey et al 2004; Beisswanger et al. 2006; Shapiro et al. 2007; Fay and Wu 2000; Hamblin and Di Rienzo 2000; Hamblin et al. 2000; Harr et al. 2002; Kauer et al. 2003; Nielsen eet al. 2005). In addition, selective sweeps have been detected in genes associated with resistance to pest control, such as chloroquine resistance in malarial parasites and warfarin resistance in rats (Kohn et al. 2000; Wootton et al. 2002), and in several genes associated with

cultivation of crop plants (Burke et al. 2005; Clark et al. 2004; Purugganan et al. 2000; Wright et al. 2005; Wang et al. 1999; Casa et al. 2006; Olsen et al. 2006).

Fixed beneficial alleles that originate as standing variation will leave a different signature following a selective sweep than that expected from a new mutation. Compared with new mutations, neutral or weakly deleterious alleles maintained as standing variation have a longer history in the population before becoming advantageous. One effect of this extra time is that it provides greater opportunity for recombination to break up the association between the soon-to-be-favored site and neutral variants at all but the nearest sites (Przeworski et al. 2005). The result is that, on average, the valley of low polymorphism that accompanies fixation of a beneficial allele will be narrower compared with that in a standard sweep (Figure 2.3).

Another effect of the greater age of standing variation compared with new mutation is the increased chance that the same beneficial allele will originate more than once on different genetic backgrounds before becoming advantageous (Hermisson and Pennings 2005, Pennings and Hermisson 2006a; Hermisson and Pennings 2006b). The result is that a sweep from standing variation will drag along more polymorphism at linked sites than will a sweep from a single new mutation, which must arise on a single background. The valley of low polymorphism characterizing a sweep from standing variation will be shallower on average compared with that of a standard sweep (Figure 2.3). Similarly, the strength of statistical associations between the selected site and nearby sites will be reduced (Przeworski et al. 2005; Hermisson and Pennings 2005). Selective sweeps from standing variation will, therefore, be weaker on average than sweeps associated with new mutations. However, if there is high mutation rate then

repeated, independent origins of the advantageous allele can occur from new mutations arising after the environmental change, producing a similar weak sweep signal (Pennings and Hermisson 2006a; Hermisson and Pennings 2006b). On the other hand, such a high mutation rate should also produce high levels of standing variation prior to the environmental change.

A possible example of a selective sweep from standing variation comes from a recent study on the *SCR* self-incompatibility locus in *Arabidopsis thaliana* (Shimizu et al. 2004). Positive selection has driven the rapid fixation of an allele that inactivates self-incompatibility at *SCR*, which encodes a cysteine-rich protein found in the pollen coat. Simulations of different historical scenarios suggest that this event occurred during the post-Pleistocene expansion of *A. thaliana* from a glacial refuge, when a scarcity of pollinators might have provided an advantage to self-pollination, despite inbreeding depression. Patterns of linkage disequilibrium around *SCR* indicate that a considerable amount of recombination occurred after the origin of the allele, but before its rapid fixation, resulting in differences in the evolutionary histories among sites in this region. As expected if the allele was present as standing variation, the selective sweep left a narrow signal in the region of DNA surrounding *SCR*. Another potential example is the *Accord* insertion associated with DDT resistance in non-African populations of *D. melanogaster.* Schlenke and Begun (2004) and Catania *et al.* (2004) found evidence that *Accord* had recently undergone a selective sweep. Although the sweep did cause a detectable reduction in polymorphism around *Accord*, the width of the region of low polymorphism was reduced relative to expectations for a region under strong selection

and of recent origin, as might be predicted if the insertion had been present as standing variation before application of DDT to the area.

A shallower and narrower selective sweep is not the only way to distinguish adaptation from standing variation and that from new mutations. A perhaps more striking difference can be found in the allele frequency spectra at neutral sites linked to the selected allele. When linked sites are found to be polymorphic following a sweep from a new mutation, they usually harbor an excess of low and high frequency alleles (Fay and Wu 2000). This is because most recombination will occur once the mutation has reached high frequency (Schlenke and Begun 2004; Kim and Nielsen 2004; Przeworski 2002; McVean 2007). Thus, recombination will usually incorporate only a few additional genetic backgrounds, each at low frequency, other than the one that first carried the beneficial allele (Figure 2.4). By contrast, recombination will put the advantageous allele on other genetic backgrounds before it becomes advantageous when a sweep originates as standing variation (Innan and Kim 2004; Przeworski et al. 2005). This can result in a more balanced genealogy when the focal allele eventually fixes, because there will be more neutral lineages associated with the fixed allele (Przeworski et al. 2005). Thus, a distinguishing feature of sweeps from standing variation is an increase in the occurrence of linked neutral sites having alleles at intermediate frequency (Figure 2.4). A potential example is the *Duffy* locus in humans, at which a null allele confers resistance to vivax malaria. This null allele is fixed in several populations exposed to malaria but is absent elsewhere. Despite evidence of a selective sweep, Hamblin and Di Rienzo (2000) did not find that diversity levels were consistently reduced, and linked sites carried more

intermediate frequency alleles than would be expected after fixation of a new beneficial allele.

Although the analysis of selective sweeps is a promising tool for detecting selection and distinguishing the origin of beneficial alleles, the approach is fraught with problems when demographic assumptions are violated (Santiago and Caballero 2005; Slatkin and Wiehe 1998; Nielsen 2001; Kim and Stephan 2002), as is often the case for natural populations. Most methods assume that populations are randomly mating and have a constant size (Maynard Smith and Haigh 1974; Beisswanger et al. 2006; Kim and Nielsen 2004; Przeworski 2002; McVean 2007; Kim and Stephan 2002; Barton 1998). Departures from these conditions can make it difficult to determine the cause of sweep patterns (but see Teshima et al. 2006). Some demographic events, such as population expansion, can lead to the same signal (e.g. an excess of rare alleles at linked neutral sites), as would positive selection on a new mutation. Other events, such as population subdivision, can distort the signal of a sweep from a new mutation and, therefore, could be confused with a sweep following selection on standing variation (e.g. both situations will result in more intermediate frequency alleles at linked neutral sites). As such, it will often be necessary to use additional complementary methods to determine whether standing variation has contributed to adaptation.

Finding the source of standing variation

Selection from standing variation can sometimes be inferred if a beneficial allele in a new environment is still present as standing variation in the ancestral population. For example, recent work on human populations has identified derived alleles that are associated with

the ability to digest lactose, the main carbohydrate present in milk (Tishkoff et al. 2007; Enattah et al. 2002). In most humans, this ability declines rapidly after weaning. However, in populations that have practiced cattle domestication, many individuals maintain the ability as adults (Swallow 2003). An allele associated with adult lactose digestion has reached high frequency in European human populations over the past 8000-9000 years, which coincides with the spread of cattle domestication from the Middle East into Europe (Beja-Pereira et al. 2003). This allele is also present in Middle Eastern populations, suggesting that standing variation from these populations probably supplied the beneficial allele along with pastoralism into Europe (Myles et al. 2005). However, this is not the whole story, because three separate alleles responsible for lactose digestion in adults arose recently in Sub-Saharan Africa and are not found elsewhere, an indication that these alleles probably arose *de novo* (Tishkoff et al. 2007; Myles et al. 2005). Thus, it is likely that both new mutations and standing variation have contributed to adaptation to pastoralism in different human populations.

A recent study by Pelz *et al.* (2005) provides another example where adaptive alleles have been found segregating in the ancestral environment. The brown rat *Rattus norvegicus* has evolved resistance to warfarin in just a few decades since the pesticide was introduced. Several different allelic variants of the gene *VKORC1* confer resistance to warfarin, and these variants are present in natural populations of brown rats throughout Europe (Pelz et al. 2005).

One of the challenges of detecting adaptation from standing variation by looking for the presence of adaptive alleles in ancestral populations is determining which population is ancestral. In the case of alleles associated with adult lactose digestion in

human populations, it was possible to use archeological and linguistic evidence to infer which populations practiced cattle domestication first, and it seems likely that these populations will also be the source of the lactose digestion allele. In other cases, geological data might be the most reliable way to determine which environment is ancestral. For instance, locations that were covered by glaciers during the last Ice Age are assumed to be new populations, whose ancestral populations resided in unglaciated areas. Another challenge is ruling out the possibility that a beneficial allele was secondarily introduced to the ancestral population by gene flow rather than having originated there. Therefore, additional evidence will be needed to confirm selection from standing variation. This problem could be resolved if several populations all independently derived from the same ancestral population are fixed for the same adaptive allele, and if gene flow is not possible directly between derived populations. Although this allele might have arisen *de novo* in one of the populations, it is unlikely to have arisen *de novo* in all of them.

Determining the history of derived alleles

The phylogenetic history of alleles can also provide evidence of adaptation from standing variation or new mutation. If a beneficial allele that has fixed in a new environment pre-dates the origin or colonization of that environment, then we can be sure that it did not arise *de novo* under the current selective conditions. For example, Colosimo *et al.* (2005) sequenced the alleles at the gene most responsible for the evolution of reduced defensive armor (bony lateral plates) in threespine stickleback *Gasterosteus aculeatus* populations that colonized freshwater from the sea at the end of the last Ice Age. The ancestral marine

population has a complete set of 32-36 bony lateral plates, whereas freshwater populations have only 0-9 plates (Figure 2.2). The same gene, *Ectodysplasin* (*Eda*), was found to be responsible for armor reduction in all freshwater populations sampled. Phylogenetic analysis of the allele sequences in the freshwater and ancestral marine populations revealed that the allele that is beneficial in freshwater originated more than two million years ago. Given that the postglacial lakes inhabited by low-armor freshwater populations have only existed for ~10,000 years, the finding implies that evolution of the low-armor phenotype has occurred by recurrent local selection on an ancient allele brought repeatedly into freshwater environments by marine founders. The allele is indeed present at low frequency in marine populations today (Colosimo et al. 2005).

The two host races of the apple maggot fly, *Rhagoletis pomonella*, also provide an example in which ancient genetic changes might have led to much more recent adaptation under novel environmental conditions (Figure 2.2). Several inversion polymorphisms have been found to be strongly associated with the length of overwintering pupal diapause in *R. pomonella* (Feder et al. 2003). This variation is significant because it differentially adapts apple and hawthorn races to the fruiting times of their hosts (Feder et al. 1997). Although the North American apple race is only 150 years old, phylogenetic analysis shows that the inversions arose at least 1.5 million years ago in Mexico (Feder et al. 2003; Michel et al. 2007) and have recently been introduced to North America by gene flow (Michel et al. 2007). The variation in diapause timing caused by these ancient inversions then contributed to the formation of an apple race adapted to the earlier fruiting time of introduced, cultivated apple orchards. Thus, life-history adaptation was

due to introgression of standing variation that predated the latest environment where it was beneficial.

Conclusions

Here we have highlighted the evolutionary patterns and consequences that occur when a population makes use of standing genetic variation rather than new mutations when adapting to a new environment. We have little information about the relative importance of these two sources of beneficial alleles after a change of environment. Nevertheless, a few case studies of ecologically relevant genes suggest that standing variation has an important role in facilitating rapid adaptation to novel environments (Steiner et al. 2007; Tishkoff et al. 2007; Pelz et al. 2005; Colosimo et al. 2005; Feder et al. 2003). The dynamics and outcome of adaptation are distinct depending on the source of variation. Understanding these differences will be integral towards predicting how populations will respond to changing environments. Rapid evolution will be necessary for the survival of many species as humans increasingly affect sudden and drastic environmental changes on the biosphere (Palumbi 2001), and this will probably be fuelled largely from standing variation.

Many questions remain about the dynamics, circumstances and consequences of adaptation from standing variation. Because most of the theory on the genetics of adaptation has focused on adaptation from new mutations, there are still gaps in our knowledge concerning the theoretical predictions when adaptation instead occurs from standing variation. Only during the past few years have alternatives to the classic theory for selective sweeps been developed for adaptation from standing variation (Przeworski

et al. 2005; Hermisson and Pennings 2005). For example, theory for the distribution of fitness effect sizes produced during adaptation from new mutations is relatively well developed, but how does this change when adapting from standing variation? We have given some reasons why this distribution of fitness effect sizes might be different from standing variation, such as the greater fixation probability of small effect alleles, but a quantitative theory for standing variation is still needed. As we accumulate further examples of adaptive alleles in natural populations, it will become possible to undertake broad comparative analyses to discover the importance of standing variation across a diverse range of taxa and conditions. By considering the unique patterns and consequences of selection on standing variation, we will gain a more general understanding of how populations adapt to novel or changing environments.

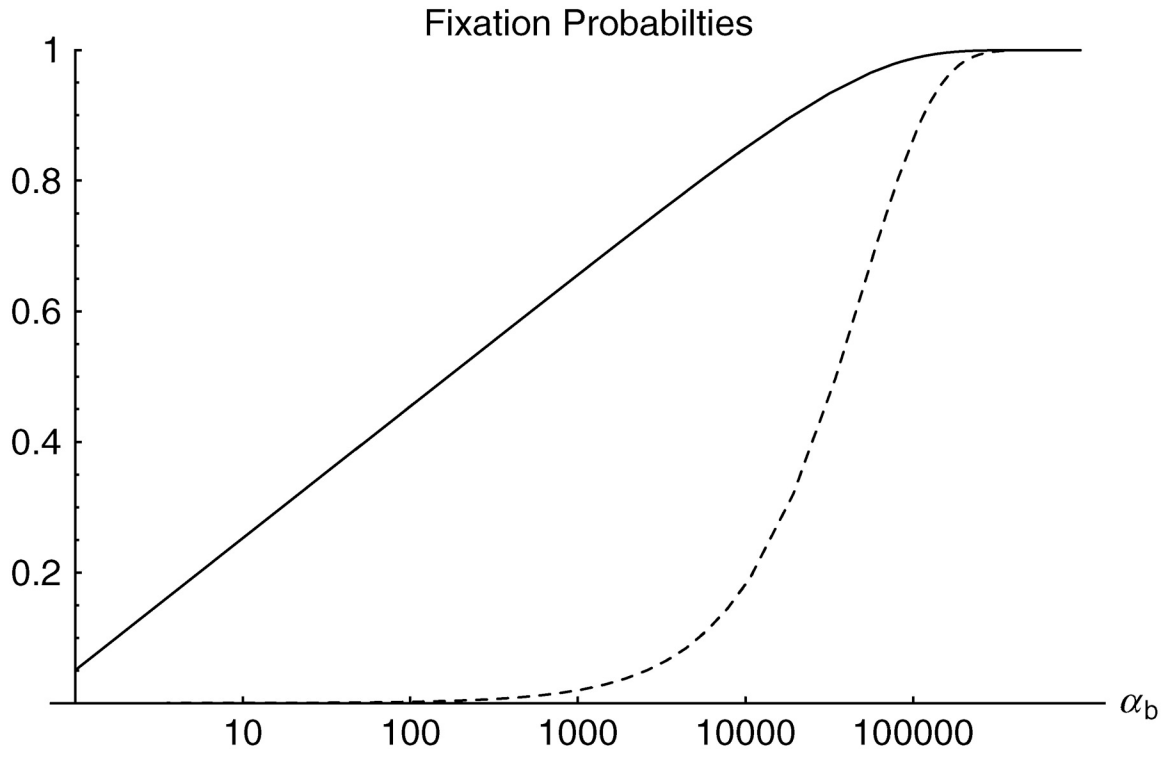

Figure 2.1. The probability of fixation of a single new mutation (dashed curve) compared with that of a polymorphic allele that arose in a single mutational event (solid curve).

$\alpha_b = 2Nesb$, where *Ne* is the effective population size and *sb* is the homozygous fitness advantage. The form of the curve for standing variation in this example assumes that N = *Ne* = 25 000, the dominance coefficient (*h*) = 0.5 and that beneficial alleles were previously neutral. α_b is plotted on a logarithmic scale. Modified with permission from Hermisson and Pennings 2005.

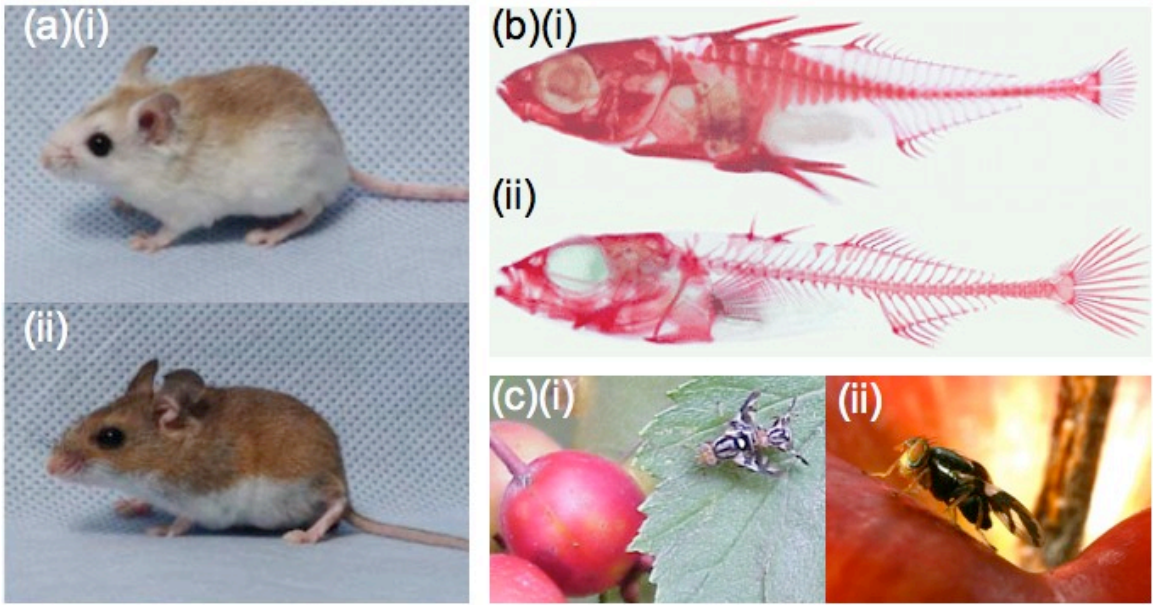

Figure 2.2 Candidates for adaptation from standing variation.
(a) *Peromycus polionotus* subspecies. The mouse in (i) is a typical Santa Rosa Island beach mouse (*P.p. leucocephalus*) and the mouse in (ii) is a typical mainland mouse (*P.p. subgriseue*). Two candidate genes, the *melanocortin-1 receptor* (*Mc1r*) and its antagonist, the *Agouti signaling protein* (*Agouti*), control most of the difference in pigmentation between subspecies. (b) Threespine stickleback *Gasterosteus aculeatus*, cleared and stained with alazarin red to highlight bone structure. The fish in (i) has many bony lateral plates, a phenotype typically found in the ocean. The fish in (ii) has many fewer plates and is typically found in freshwater lakes. (c) Apple maggot fly *Rhagoletis pomonella*. The flies in (i) are from a host race specialized to feed on hawthorn. The fly in (ii) is from a host race specialized to feed on apple. Standing variation originating in Mexico is implicated in the evolution of overwintering pupal diapause in the apple race. Reproduced with permission from H.E. Hoekstra (a) and J.L. Feder (c).

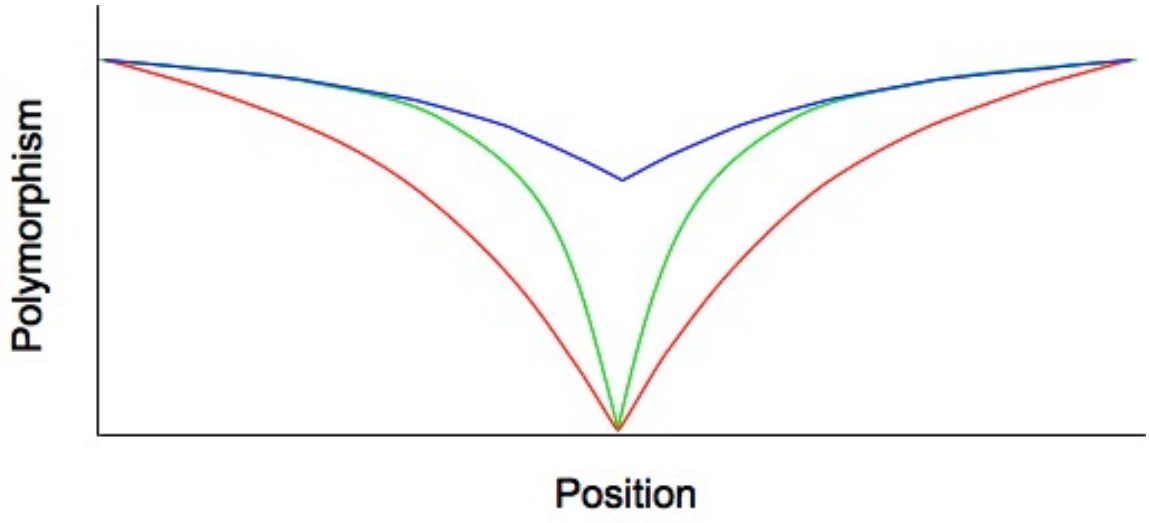

Figure 2.3. A schematic of differences between standing variation and new mutation in the expected signature of selection around a recently fixed beneficial allele (site at center of figure).

Fixation of a new mutation eliminates polymorphism near the site (red lines) because the advantageous allele is linked from its time of origin to a single set of neutral variants nearby. Fixation of an allele present as standing variation can result in a narrower region of reduced polymorphism than in the case of a new mutation because its greater age has exposed it to more recombination events with nearby neutral sites before the selective period (green lines). Standing variation might also include multiple alleles that have arisen independently on different genetic backgrounds, in which case polymorphism will not be reduced as much in the selected region (blue lines).

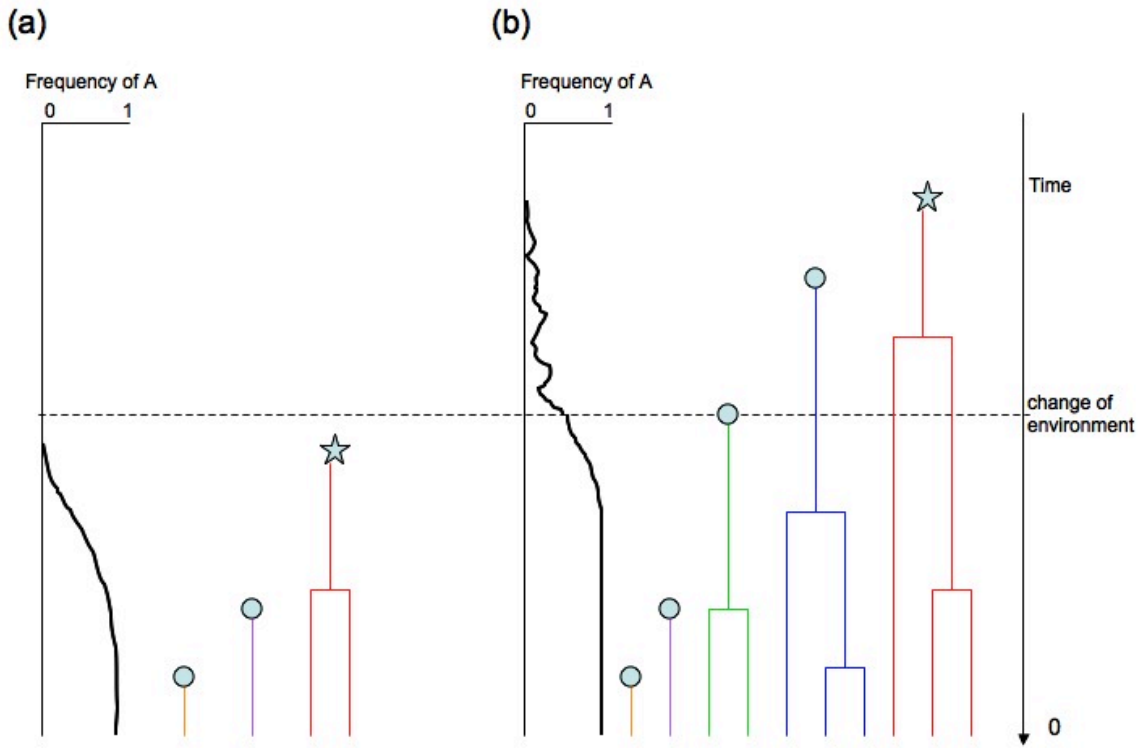

Figure 2.4. Genealogical trees of a segment of neutral DNA sequence linked to a beneficial allele that has fixed from a new mutation (a) and from standing variation (b).

The star in each panel designates the time of unique origin of the favored allele, A. The subsequent frequency of the allele through time is illustrated on the left of each panel (thick black lines). As A increases in frequency, neutral mutations arise in the segment, leading to diversification of lineages (red lineages). Also, recombination events (indicated by filled circles) might link additional, independent neutral lineages with the favored allele (other colors). At fixation, these new lineages will be present at low frequency in the new mutation scenario, but might be at intermediate frequency in the standing variation scenario because they had more time to become associated with A when it was still relatively rare.

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3. NATURAL SELECTION ON A MAJOR ARMOR GENE IN STICKLEBACK[2]

Introduction, Results and Discussion

Adaptive evolution occurs when genetic variation affects phenotypes under selection. This process has been detected by the discovery of candidate genes underlying phenotypic traits whose adaptive significance is known or suspected (Colosimo et al. 2005; Abzhanov et al. 2004; Albertson et al. 2005; Bradshaw et al. 1998; Hoekstra et al. 2006; Shapiro et al. 2004; Rogers and Bernatchez 2007) and by identifying statistical signatures of selection on genomic regions affecting phenotypic traits (Akey et al. 2004; Nielsen et al. 2005; Nielsen et al. 2005; Shapiro et al. 2007; Wootton et al. 2002; Wright et al. 2005). However, field experiments evaluating the fitness consequences of allelic substitutions at candidate loci should provide estimates of the timing and strength of selection, enhance understanding of the genetics of adaptation and yield insights into the mechanisms driving changes in gene frequency.

Freshwater threespine sticklebacks (*Gasterosteus aculeatus*) originated from marine populations that invaded newly created coastal lakes and streams throughout the northern hemisphere following the last ice age. Within the past 20,000 years or less, freshwater populations repeatedly underwent a loss in bony armor plating (Bell and Foster 2004). Marine sticklebacks are typically armored with a continuous row of 30 to 36 bony lateral plates on each side (complete morph), whereas freshwater sticklebacks typically possess 0 to 9 plates (low morph) or, less often, an intermediate number of

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plates (partial morph) (Bell and Foster 2004; Bell 1977; Hagen and Gilbertson 1973) (Figure 3.1). Armor reduction following colonization of freshwater evolved rapidly (Klepaker 1993; Kristjansson et al. 2002; Kristjansson 2005; Bell et al. 2004) from the fixation of a clade of low alleles of the *Ectodysplasin* gene (hereafter the *Eda* low allele). This allele evolved approximately 2 million years ago and is rare (~1%) in the ocean (Colosimo et al. 2005). The repeated fixation of this allele implies that it undergoes positive selection in freshwater, because genetic drift alone is unlikely to produce a strong correlation between phenotype and environment (Simpson 1953; Rundle et al. 2000; Schluter et al. 2004).

Fish with reduced armor have a juvenile growth advantage (Marchinko and Schluter 2007), which may result from the higher cost of mineralizing bone in freshwater (Giles 1983; Bell et al. 1993), which has low ion concentrations relative to marine environments. This increased growth rate should, in turn, reduce predation by insects (Foster et al. 1988), and increase lipid stores, resulting in higher over-winter survival (Curry et al. 2005). Larger fish also may breed earlier (Schultz et al. 1991), have access to better territories, an increase in mating success and a higher reproductive output (Schultz et al. 1991; Candolin and Voigt 2003; Einum and Fleming 2000; Hasselquist 1998; Aebisher et al. 1996; Moller 1994; Rowe et al. 1994; Landa 1992; Verhulst and Tinbergen 1991). To test this hypothesis, we tracked adaptive evolution at the *Eda* locus in replicated transplants of marine stickleback to freshwater environments. We predicted that we would observe positive selection on the low allele via advantages in growth, survival, and reproduction. We also looked for deviations from this expectation, which might suggest that *Eda* or linked genes have unexpected fitness effects.

We experimentally introduced adult wild marine fish heterozygous at the *Eda* locus to four freshwater ponds. The fish were trapped from a marine stickleback population in south-western British Columbia. We introduced approximately equal numbers of these fish (n = 45-46) to each pond in the spring of 2006, initiating replicate freshwater invasions. Within 60 days we observed larval fish in each colonized pond indicating that the marine colonizers were breeding. Genotyping of four microsatellite markers, which were all in linkage equilibrium with *Eda*, confirmed that nearly all alleles present in the parents were at similar frequencies in the progeny (Figure A.1), suggesting that founding events did not confer any sampling artifacts. Genotype frequencies at *Eda* in the F1 generation were not significantly different from the predicted 1:2:1 ratio (Figure 3.2A, pond 1: $\chi^2 = 0.06$, df = 2, $P = 0.97$, pond 2: $\chi^2 = 1.09$, df = 2, $P = 0.58$, pond 3: $\chi^2 = 1.09$, df = 2, $P = 0.58$, pond 4: $\chi^2 = 1.20$, df = 2, $P = 0.55$). Subsequently, we sampled 50 fish from each pond ten times over one-year to monitor changes in offspring allele frequencies.

We observed strong fluctuations in *Eda* allele and genotype frequencies, with replicate ponds showing nearly parallel oscillations (Figure 3.2A). We did not observe strong changes in allele frequency in the unlinked microsatellite markers, suggesting that these results are not due to demographic effects (Figure A.1). Fish achieved their adult number of lateral plates after reaching a standard length of ~30 mm (Bell et al. 1993; Bell 2001; Bell 1981). Most experimental fish passed this threshold between October and November 2006 [average length in October was 27.32 mm (± 5.99 SD), average length in November was 33.14 mm (± 4.70 SD)]. In agreement with our predictions for growth, by October juvenile fish carrying the low allele were larger than juvenile fish homozygous

for the complete allele. Mean body length was positively associated with the number of low alleles per genotype in all ponds (one-tailed *t*-test of four slopes, $t = 2.53$, df = 3, $P = 0.043$). We also noted higher over wintering survival rates in fish with the low allele. From October 2006 to May 2007, the frequency of the complete allele dropped from 67% to 49%, reflecting the comparatively poor survival of individuals homozygous for the complete allele. We calculated that the selection coefficient (*S*) against the complete allele between these dates was 0.52 (± 0.10 SEM) (Figure 3.2; see methods).

At the start of the breeding season in May 2007, the number of low alleles carried by an individual was again positively associated with body length in all ponds (one-tailed *t*-test of four slopes, $t = 2.35$, df = 3, $P = 0.050$), and sexually mature individuals were significantly larger than non-breeding individuals (Figure 3.3, Welch two-tailed *t*-tests, pond 1: $t = 2.47$, df = 6, $P = 0.049$, pond 2: $t = 9.40$, df = 2, $P = 0.006$, pond 3: $t = 2.61$, df = 9, $P = 0.027$, pond 4: $t = 4.23$, df = 13, $P < 0.001$). The genotypes of the earliest reproductive individuals were biased towards carrying the low allele compared with non-reproductive individuals, with 95% being heterozygous or homozygous low (Figure 3.3; tested by the interaction between breeding status and genotype in a log-linear model, $\chi^2 = 7.30$, df = 2, $P = 0.026$; no effects of pond were detected, $\chi^2 = 2.88$, df = 6, $P = 0.82$). By July 2007 most individuals had reached sexual maturity, and we observed little difference in genotype frequencies between sexually mature individuals and the overall population (Figure 3.3; $\chi^2 = 2.56$, df = 2, $P = 0.28$). By this time we also could not detect a correlation between size and *Eda* genotype ($t = -0.30$, df = 3, $P = 0.607$). In all four ponds, the frequency of the low allele was greater in the first sample of F2 offspring in June 2007 than in all F1 adults sampled in May [June F2: 57.0% (± 4.1% SEM), May F1:

51.6% (± 1.4% SEM), Fig. 2A, one tailed *t*-test, $t = 2.14$, df = 3, $P = 0.061$]. By July the frequency of the low allele in F2 juveniles had decreased to 52.2% (± 3.7% SEM), reflecting the similar genotypic ratios of breeding and nonbreeding adults later in the breeding season.

These patterns linking the low *Eda* allele with higher growth, improved survival and earlier breeding, are consistent with the hypothesis that positive selection stemmed from a reduced burden of producing armor plates in freshwater. This effect, combined with the possibility of reduced vertebrate predation pressure in freshwater compared to the sea (Bell et al. 1993; Reimchen 2000), may account for the evolution of low plated populations in freshwater. At the same time, selection against plate production does not fully explain the observed changes in *Eda* allele frequencies. We noted selection favoring the complete allele in all four ponds (Figure 3.2A) very early in life, before the fish attain the size at which number of lateral plates is finalized (about 30 mm). The calculated selection coefficient (*S*) against the low allele between July and October 2006 was 0.50 (± 0.16 SEM; Figure 3.2C), which offset the gains occurring later in life. We also observed oscillations in the relative fitness of heterozygotes at *Eda*, which are difficult to explain solely in terms of the burden of lateral plates because the size and number of plates in heterozygotes are intermediate between low and complete homozygotes (Schluter et al. 2004). The decline in low *Eda* allele frequencies early in life was associated with a drop in the frequency of heterozygous fish and a rise in the frequency of the homozygous complete genotype, suggesting that there is heterozygote underdominance for fitness at this stage [$h = -1.38$ (± 0.23 SEM)]. Underdominance was especially apparent by October 2006, when heterozygous fish made up less than 1/4 of

the total in our samples instead of the 50% observed at the start of the F1 cohort. This episode was followed by a period between November 2006 and May 2007 during which the heterozygotes at *Eda* had the highest fitness of all three genotypes [$h = 2.57$ ($\pm$ 0.98 SEM)]. Although positive selection favored the low allele during this period, heterozygotes increased in frequency much faster than the homozygous low genotype (Figure 3.2C). These findings suggest that either variation at the *Eda* gene has direct or epistatic effects on other phenotypic traits contributing to fitness, or it is linked to another, unidentified locus affecting fitness.

Our results highlight the utility of direct measurements of natural selection on genes for understanding the genetic basis of adaptation by enabling us to test a mechanism favoring reduction of lateral plates in freshwater environments. Many of our results are consistent with selection against high plate number, although they do not rule out the possibility that selection is also occurring on genes tightly linked to *Eda* (Colosimo et al. 2005). Our results also expose opposing selection on *Eda* early in life similar in magnitude to the measured advantage of the low allele later in life. This demonstrates not only that countervailing selection pressures diminish the advantage of the low allele over the whole life span, but also that the overall fitness effects of *Eda* do not seem to be determined solely by differences in lateral plate number. Along with the fluctuating dominance in fitness at the *Eda* locus, these results indicate that there may be additional pleiotropic effects of this gene. This work underscores the need for a synthesis of population biology and genomics, to determine the genetic basis of fitness differences in natural populations (Ellegren and Sheldon 2008).

Methods

Collection of experimental fish

We collected stickleback in April and May of 2006 from Oyster Lagoon on the Sechelt peninsula in western British Columbia (49°36'48.6" N, 124°1'46.88" W). Oyster lagoon is a saltwater inlet with salinity ranging from 28-32 ppt, in which phenotypically partially armored fish occur at an approximate frequency of 0.01. This population breeds in saltwater and the rare sticklebacks with reduced plate number are marine in all other phenotypic characteristics (shape, size, color, spine length; Foster 1994; Foster et al 2008; Siamoto 1995). We sampled approximately 35,000 fish using unbaited minnow traps. We brought all fish not possessing the full number of lateral plates (n = 354) back to the lab, including those missing only one or two lateral plates, and released all other captured fish. In the lab we injected individual fish subepidermally with a fluorescent visible implant elastomer tag (Northwest Marine Technology) using a 29 gauge syringe.

A small fin clip taken from each fish allowed us to genotype the fish at diagnostic loci within the *Eda* gene that distinguish between low and complete alleles (Colosimo et al. 2005). We also genotyped fish at a SNP within an α1 subunit of Na+-K+-ATPase for freshwater versus marine residency (Jones et al. 2006) to confirm that they were marine residents rather than recent freshwater or stream migrants. Almost all alleles (96%) at this marker were consistent with those observed in marine populations.

Pond sampling

Of the 354 partial morphs collected from Oyster Lagoon, 182 were heterozygous at *Eda*. On June 1, 2006, we released the heterozygous fish into 4 artificial ponds located at the

University of British Columbia, Vancouver, British Columbia, Canada (pond 1 $N = 45$, pond 2 $N = 46$, pond 3 $N = 45$, pond 4 $N = 46$). These ponds measure 23 m × 23 m and have a maximum depth of 3 m in the centre, as described (Schluter 1994). Like many coastal lakes in British Columbia, the ponds are lined with sand and bordered with limestone. All ponds had been previously drained, cleaned and refilled in 2001, allowing plant and invertebrate communities to re-establish, but remaining free of fish until this experiment. The plants and invertebrates used to seed the ponds were collected from Paxton Lake, Texada Island, British Columbia, an 11-ha lake that contains wild sticklebacks. Apart from their construction, initialization, and use in prior experiments, the ponds are unmanipulated environments. In previous experiments these ponds have sustained large populations of sticklebacks over multiple generations, with life cycles and diets characteristic of their wild source populations (Schluter 2003). Growth rates of fish in the ponds are similar to those of wild fish in freshwater lakes (Day et al. 1994). We observed F1 progeny in the ponds in August 2006. We were able to distinguish between the F1 fish and their parents because parents retained their elastomer tags and were significantly larger in size. The colonizing fish accounted for a very small proportion of the overall populations following the F1 generation, and we only observed three colonizers out of ~1500 individuals sampled over the full duration of the experiment. No colonizers were caught after the October 2006 sample. Sampling of the separate cohorts was not possible after July 2007 because F1 and F2 generations were no longer distinguishable, as their body size distributions had merged. Fish were sampled with dip nets, traps, or a seine net. A variety of sampling methods were necessary during different times of the year because small juveniles cannot be caught in traps, the seine net disturbs

nesting sites, and large adults cannot be easily caught with dip nets. We detected no difference in genotype frequencies using these different sampling methods as genotype ratios in samples from the same ponds, in the same month, with different methods were not significantly different (pond 1: $\chi^2 = 0.37$, df = 2, $P = 0.83$, pond 2: $\chi^2 = 1.73$, df = 2, $P = 0.42$, pond 3: $\chi^2 = 2.08$, df = 2, $P = 0.35$, pond 4: $\chi^2 = 0.04$, df = 2, $P = 0.98$). We recorded total length, phenotype, and breeding condition for all fish sampled, and then preserved each individual at -80^{o}C.

Genotyping

We isolated total genomic DNA from small caudal fin clips using a standard proteinase K phenol chloroform protocol (Sambrook et al. 1989). We quantified DNA yield using spectrophotometry and then preserved DNA samples at -20°C. We used diagnostic in/del loci to identify low and complete *Eda* alleles (isolated from loci Stn380 and Stn381 within introns two and six of the *Eda* gene on linkage group 4, respectively (Colosimo et al. 2005)). Four unlinked microsatellite loci, isolated and characterized by the Stanford Genome Research Centre, were selected for additional population genetic analyses (Linkage group and Genbank Accession numbers in parentheses): Stn224 (LG 11, BV678144), Stn314 (LG 8, BV678119), Stn387 (LG 2, BV678140), and Stn388 (LG 9, BV678141). Microsatellite and *Eda* alleles were amplified by PCR with a DNA Engine® Peltier Thermal Cycler (MJ research, Inc., Waltham, Massachusetts) in 10 µl reactions containing 5 to 15 ng of genomic DNA, 1uM of each forward and reverse primer, 1X PCR buffer, 0.25 mM of each dNTP, 1.5 mM MgCl2, and 0.25U of AmpliTaq Gold polymerase (Applied Biosystems, Foster city, California). Cycling conditions were

standardized over all loci as follows: 93 ^{o}C for 3 min, 95 ^{o}C 30 s, 59 ^{o}C 30s, 72 ^{o}C 30 s, 5 cycles of 94 ^{o}C 30 s, 59 ^{o}C 30 s, 72 ^{o}C 30 s, 35 cycles of 90 ^{o}C 30 s, 60 ^{o}C 30 s, 72 ^{o}C 30 s, followed by 72 oC for 10 min and then cooled to 4 ^{o}C. Electrophoresis consisted of pooling PCR products with an internal size standard (LIZ 500bp, Applied Biosystems) and loading onto a 3730S Automated Sequencer (Applied Biosystems). Allelic sizes (in base pairs) were determined by reference to the internal sizing standard in the software GENEMAPPER (Applied Biosystems). We found no significant difference between genotype frequencies at Stn380 and Stn381 in the first four samples (n = 698, χ^2 = 0.275, df = 2, P = 0.872) and therefore used only Stn381 for the remaining samples. All genotype data presented is for Stn381 only.

Calculating selection and dominance coefficients

We calculated S (the selection coefficient) for viability selection on the low and complete *Eda* alleles as the change in frequency of each allele relative to the change in frequency of the most fit allele, subtracted from 1 (Hartl and Clark 1997). Similarly, S for *Eda* genotypes was calculated as the change in frequency of the homozygous low genotype relative to the change in frequency of the homozygous complete genotype, subtracted from 1 when selection favored the homozygous complete genotype. When there was selection against the homozygous complete genotype we calculated S as the change in frequency of the homozygous low genotype relative to the change in frequency of the homozygous complete genotype minus 1. We calculated h (the dominance coefficient) as the change in frequency of the heterozygous genotype relative to the change in frequency

of the homozygous complete genotype minus 1, divided by *S* (Hartl and Clark 1997). Standard errors for *S* and *h* are from measurements of $n = 4$ ponds.

Testing Hardy-Weinberg proportions with combined neutral markers

Departures from Hardy-Weinberg proportions were tested in each sample with an approximation of an exact test from a Markov chain iteration implemented in GENEPOP 4.0 (Raymond and Rousset 1995). Multilocus values of significance for HW tests were calculated following Fisher's method to combine probabilities from different tests (Sokal and Rohlf 1995). Critical significance levels were corrected for multiple tests following the Bonferroni procedure (Rice 1989).

Figure 3.1. Lateral plate morphs in marine stickleback.

Complete morph (top), partial morph (middle), and low morph (bottom). Fish were stained with Alazarin red to highlight bone.

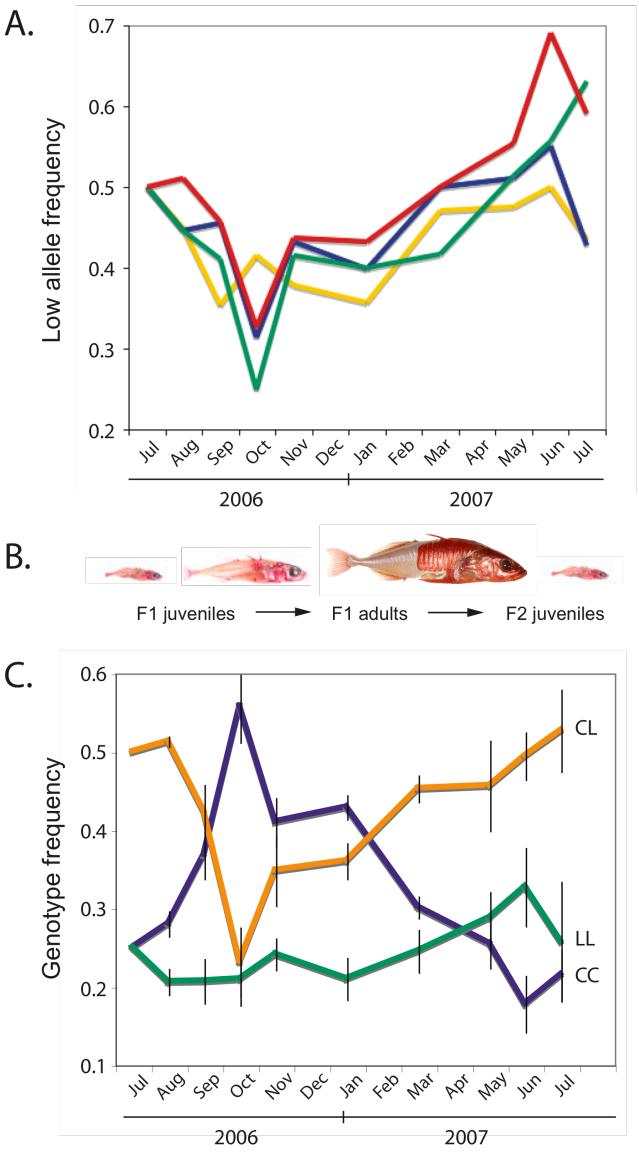

Figure 3.2. Selection on Eda genotypes.

(A) Frequency of the low allele in four replicate ponds (different colored lines). All samples are from the first (F1) cohort of offspring except the June and July 2007 samples, which are from the second (F2) pond generation. (B) Approximate life history stages through the course of the experiment. Fish stained as in Fig 1. (C) Genotype frequencies averaged across all four ponds. All samples are as in 2A. Purple = homozygous complete genotype (CC), orange = heterozygote genotype (CL), and green = homozygous low genotype (LL). Vertical bars show standard errors on the basis of $n = 4$ ponds.

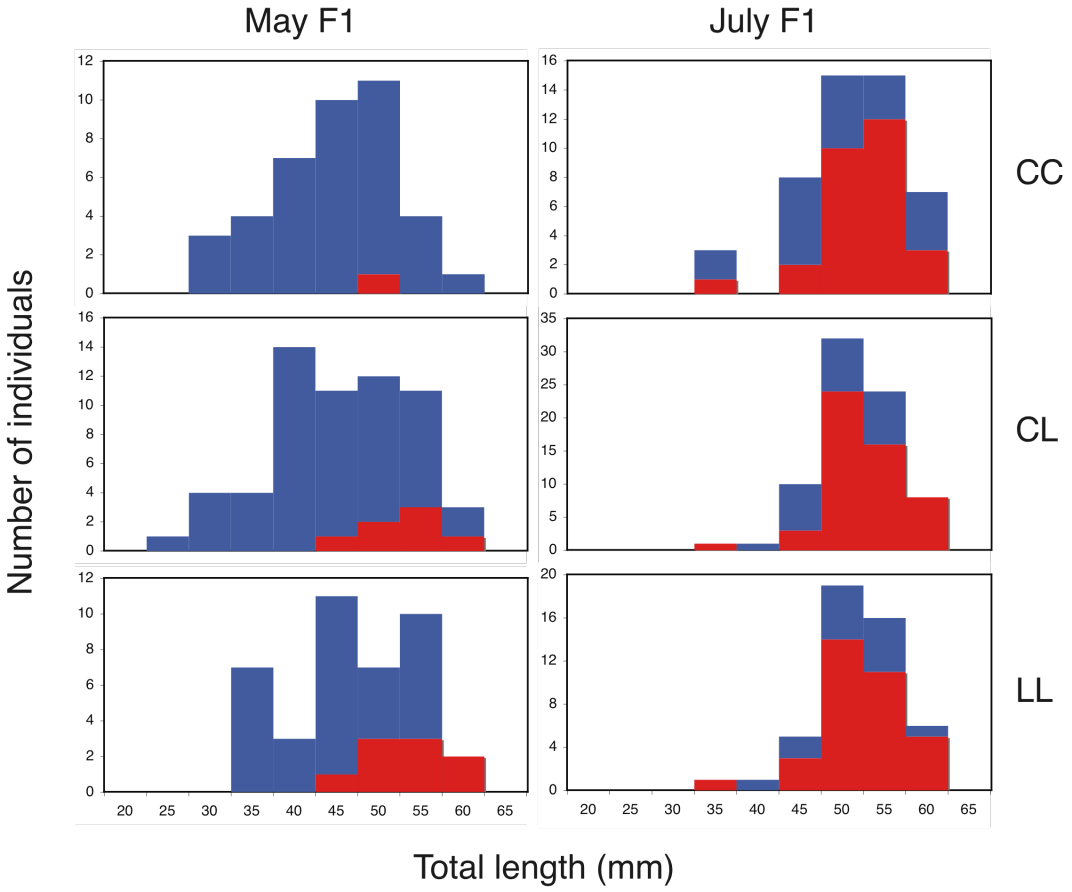

Figure 3.3. Body length of individuals in the first (F1) pond cohort during the breeding season, in May and July 2007 summed across all ponds.

Red = individuals in reproductive condition; blue = individuals not in reproductive condition. *Eda* genotypes are labeled on the right axis: homozygous complete (CC), heterozygous (CL), and homozygous low (LL).

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4. ENVIRONMENT SPECIFIC PLEIOTROPY FACILITATES DIVERSIFICATION AT THE ECTODYSPLASIN LOCUS IN THREESPINE STICKLEBACK [3]

Introduction

A major goal of evolutionary biology is elucidating the mechanisms responsible for patterns of diversity in nature. Divergent natural selection between populations in different environments has been repeatedly demonstrated to be the primary driver of phenotypic variation (Schluter 2000; Benkman 2003; Rundle and Nosil 2005; Grant and Grant 2008;). However, the potential for natural selection to shape patterns of variation may be influenced by developmental constraints that prevent all possible phenotypic variants from being produced. One way that these constraints can arise is through pleiotropy, i.e. single genes with effects on more than one trait under selection. If there is antagonistic pleiotropy, such that selection on different traits favors different alleles at a gene, then much greater strength of selection is required to produce the same rate and direction of evolution than would be needed if the traits were genetically independent (Lande 1979). While the importance of pleiotropy for evolutionary processes is well recognized (Barton 1990; Keightley and Hill 1990; Otto 2004), we have little understanding of how these genetic effects are mediated by ecological context. Studies investigating the pleiotropic effects of candidate genes have typically been conducted under uniform conditions (Doebley et al. 1997; Nesbitt and Tanksley 2001; Beldade et al. 2002; Bomblies and Doebley 2006; Kronforst et al. 2006; Lattorff et al. 2007; Wagner et al. 2008). The few studies that have tested for pleiotropic effects in varying environments

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provide evidence that gene by environment interaction can strongly influence the extent of pleiotropy (Lukens and Doebley 1999; Scarcelli et al. 2007; Mensch et al. 2008). Thus, for a comprehensive understanding of the relationship between molecular variation and fitness it is essential that we determine the pleiotropic effects of adaptive genes and the interaction of these effects with the environment.

Genes that produce specific phenotypes in particular environments provide good candidates for studying the genetics of adaptive change. The *Ectodysplasin* (*Eda*) locus controls the majority of variation (~75%) in bony lateral plates between marine and freshwater threespine stickleback, with the number of lateral plates in freshwater populations greatly reduced relative to marine populations (Colosimo et al. 2005; Makinen et al. 2008). Fish homozygous for "complete" alleles typically possess a row of 30 to 36 plates (complete morph), whereas a majority of homozygotes for "low" alleles possess 0 to 9 plates (low morph). Most heterozygotes possess an intermediate number of plates (partial morph) (Hagen and Gilbertson 1972; Bell 1977; Bell and Foster 1994). Lateral plates play a defensive role in stickleback, not only increasing the difficulty of ingestion by predatory vertebrates (Reimchen 1983), but also improving the probability of escape and survival after capture (Reimchen 1992, 2000). It is thought that the complete allele will be favored in oceanic habitats, where sticklebacks are often far from cover and experience intense vertebrate predation pressure (Reimchen 2000; Bell 2001; Colosimo et al. 2004; Marchinko 2008). In contrast, the presence of lateral plates may be a disadvantage in freshwater, where sticklebacks are closer to cover, and acceleration and maneuverability for escape may be more important than survival after capture (Reimchen 2000; Bell 2001; Bergstrom 2002). Thus, the primary hypothesis for the distribution of

Eda alleles found between environments is that different predation regimes lead to divergent selection on armor.

An alternative hypothesis is that when marine sticklebacks invade freshwater environments natural selection favors the low allele because it confers an advantage in growth rate. Marchinko and Schluter found that reduced armor phenotypes grew more quickly than fully armoured phenotypes when raised in freshwater in a laboratory experiment, although the specific genotypes at *Eda* were unknown (Marchinko and Schluter 2007). A recent field study has found evidence that fish carrying the *Eda* low allele gain a growth advantage that leads to higher overwinter survival and reproductive success (Barrett et al. 2008). These findings suggest that *Eda* may have pleiotropic effects on armor and growth in freshwater. These effects might be antagonistic if reduced ion concentrations in freshwater create a developmental constraint that prevents maximizing both armor and growth (Giles 1983; Bell et al. 1993; Arendt et al. 2001). Under this hypothesis, the evolution of low plated populations in freshwater environments may be the result of a correlated response to positive selection for increased growth rate, rather than negative selection on armor (Marchinko and Schluter 2007).

We tested these hypotheses by comparing pleiotropy of *Eda* in both salt and fresh water in a laboratory experiment. We measured growth rate and armor phenotype of F1 offspring produced from crosses of wild marine fish that are heterozygous at the *Eda* locus, having one complete and one low allele. Because these fish are otherwise marine in their genetic makeup, our crosses minimize the influence of other genes that differentiate marine and freshwater populations, with the possible exception of genes closely linked to *Eda* (Colosimo et al. 2005; Miller et al. 2007). Antagonistic pleiotropy

is central to the study of life history evolution (Leroi 2001) and when combined with gene by environment interaction can lead to the maintenance of genetic diversity (Barton 1990). The presence of environment specific pleiotropic effects may help to provide an explanation for the maintenance of standing variation at the *Eda* locus (Colosimo et al. 2005).

Methods

Sample populations

We collected marine sticklebacks in April and May of 2006 from Oyster Lagoon on the Sechelt peninsula in western British Columbia (49°36'48.6" N, 124°1'46.88" W). Oyster lagoon is a saltwater inlet with salinity ranging from 28-32 ppt, in which phenotypically partial fish occur at an approximate frequency of 0.01. We sampled approximately 10,000 fish using minnow traps. We kept partially plated fish, including those missing only one or two lateral plates, and returned all other captured fish to the lagoon. To allow individual identification, we injected each fish subepidermally with a fluorescent visible implant elastomer tag (Northwest Marine Technology) using a 29 gauge syringe.

Genotyping

We genotyped fish at a diagnostic locus within the *Eda* gene that distinguishes between low and complete morph alleles. We isolated total genomic DNA from small caudal fin clips using a standard proteinase K phenol chloroform protocol (Sambrook et al. 1989). We quantified DNA yield using spectrophotometry and then preserved DNA samples at -20°C. We used diagnostic indel loci to identify low and complete *Eda* alleles (isolated

from loci Stn381 within intron six of the *Eda* gene (Colosimo et al. 2005)). *Eda* alleles were amplified by PCR using a DNA Engine® Peltier Thermal Cycler (MJ research, Inc.) in 10 ul reactions containing 5 to 15 ng of genomic DNA, 1uM of each forward and reverse primer, 1X PCR buffer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.25U of AmpliTaq Gold polymerase (Applied Biosystems). Cycling conditions were standardized over all loci as follows: 93 ^{o}C for 3 min, 95 ^{o}C 30 s, 59 ^{o}C 30 s, 72 ^{o}C 30 s, 5 cycles of 94 ^{o}C 30 s, 59 ^{o}C 30 s, 72 ^{o}C 30 s, 35 cycles of 90 ^{o}C 30 s, 60 ^{o}C 30 s, 72 ^{o}C 30 s, followed by 72 ^{o}C for 10 min and then cooled to 4 ^{o}C. Electrophoresis consisted of pooling PCR products with an internal size standard (LIZ 500bp, Applied Biosystems) and loading onto the Applied Biosystems 3730S Automated Sequencer. Allelic sizes (in base pairs) were determined by reference to the internal sizing standard in the software GENEMAPPER (Applied Biosystems).

Crossing design

Using artificial fertilization, we made 24 families from 18 females and 18 males caught from Oyster lagoon. We never crossed a female to the same male twice. Because all parents are heterozygous, the ratio of *Eda* genotypes in the progeny should be 1:2:1 homozygous complete: heterozygous: homozygous low. To make a cross, we first equally distributed a female's eggs into two separate Petri dishes. One Petri dish contained fresh water (0 ppt) and the other contained artificial salt water (30 ppt; Instant Ocean synthetic seasalt, Aquarium Systems, Inc., Mentor, OH) both at a pH of 7. We then sacrificed a male using MS-222 and removed both testes. We divided one testis in half and placed each half in one of the Petri dishes. The second testis was preserved in Ginzberg solution

(Hart and Messina 1972) for use in a future cross. We crushed the testis placed in Petri dishes to release sperm. We left the half clutches of eggs and the sperm for 20 minutes and then placed them into separate plastic egg cups (pint cups with fine fiberglass mesh lining the bottom) and submerged each into a separate egg tank (20 L) according to salinity treatment. We added methylene blue to egg tanks to reduce fungal growth and removed any eggs that became inviable due to fungal growth. Eggs remained in aerated egg-tanks for eight days, and then we transferred them to 102 L tanks with the appropriate salinity treatment. We placed up to five half clutches from crosses made within three days of each other into each tank and after eggs hatched and larvae dropped into the tanks, we removed the cups and any unhatched eggs.

Experimental rearing

We fed surviving larvae live brine shrimp twice per day for six weeks and then fed each tank one 3.5 oz cube of frozen *Daphnia* once per day until 12 weeks of age, followed by a blood worm diet. After feeding stopped we removed any remaining food by filtration or manual siphoning, ensuring that each individual was fed to satiation. After five weeks we distributed the individuals in each tank equally among four 102 L tanks connected by a water circulation system, with no tank containing more than 24 individuals. Each group of four interconnected tanks thus contains the progeny of no more than five families with similar hatching dates and constitutes a separate experimental block in our statistical analysis. We gave each block a salinity treatment identical to that in which the corresponding fish introduced to it were previously raised. We conducted the experiment in two environmental chambers, with four blocks (two saltwater and two freshwater) per

chamber.

When fish reached a mean length of 25 mm from snout to the tip of the caudal peduncle (standard length), we injected each individual subepidermally with a fluorescent visible implant elastomer tag (Northwest Marine Technology) using a 29 gauge syringe, and genotyped the fish at Stn381. Each fish was given a tag that distinguished it from all other fish in the same tank. Thus, we were able to record individual survival and growth throughout the experiment. Juvenile growth rate, defined as growth rate before plates were fully developed, was calculated as the standard length divided by the number of days since the mean hatch date of all the fish in the corresponding block (hatch dates within blocks did not vary by more than three days). The average standard length of the juvenile fish over all blocks was 27mm. Total growth rate was recorded when we observed the first individuals coming into reproductive condition, approximately nine months after the juvenile growth rate measurement was obtained, and was calculated as standard length divided by the number of days since the mean hatch date of all fish in the corresponding block. Adult growth rate was calculated as the difference in standard length between the total and juvenile length measurements, divided by the number of days since the juvenile measurement. Because of mortality during the experiment, we obtained fewer total growth rate measurements than juvenile growth rate measurements. These deaths did not result in any significant change in genotype ratios during the experiment ($\chi^2 = 0.921$, df = 2, $P = 0.631$).

Analyses

Our main objective was to determine whether there was an association between *Eda*

genotype and growth rate and if this association differed between the extremes of salinity experienced by sticklebacks in nature. To test the influence of genotype on growth rates, we used linear models in R 2.7.0 (R Core Development Team 2008) to obtain the slope coefficient from the linear regression of the number of low alleles per individual against growth rate separately for each block. Each linear model contained terms for the number of low alleles (0,1,2) and the heterozygosity (0,1) of individuals in a block (the heterozygosity term was included to represent dominance). We then employed a linear mixed effects model in R 2.7.0 (R Core Development Team 2008) to test for an effect of salinity on the slopes. Blocks were treated as random effects nested within random chamber effects. To investigate the role of ontogeny, we calculated the difference in slope coefficients for juvenile and adult growth rates in each block and tested for differences between salinity treatments using the same linear mixed effect model approach as was used for the separate juvenile and total growth analyses. A significant effect of salinity in this model indicates that the effect of salinity on the relationship between genotype and growth differs between ontogenetic stages. We repeated these analyses on the heterozygosity coefficients from the block linear models but do not present them because neither the mean nor the effect of salinity was large or significant for any growth stage.

Results

We found a strong association between plate phenotype and *Eda* genotype in both environments (freshwater: Goodman and Kruskal Υ=0.70, n = 187, χ^2 = 128.7, d.f. = 4, $p < 10^{-15}$, saltwater: Υ=0.73, n = 126, χ^2 = 105.9, d.f. = 4, $p < 10^{-15}$, combined: Υ=0.70, n = 313, χ^2 = 188.7, d.f. = 4, $p < 10^{-15}$, Table 4.1, Table B.1). Because *Eda* genotype

strongly predicts plate phenotype regardless of environment, we investigated the effect of environment on pleiotropy by testing its effects on the association between *Eda* genotype and growth rate. In each experimental block we obtained the slopes of linear regressions of final growth rate on the number of low alleles possessed by an individual (0 for homozygous complete, 1 for heterozygote, 2 for homozygous low). We found that individual growth rates were positively associated with the number of low alleles at the *Eda* locus in freshwater (Figure 4.1A, mean of block slope coefficients = 0.0030; SE = 0.0016). In contrast, there was no association between growth rate and genotype in saltwater (Figure 4.1A, mean of block slope coefficients = -0.0010; SE = 0.0015). We detected a marginally significant difference between the mean slope coefficients in freshwater and saltwater treatments (linear mixed effects model on block slope coefficients, salinity: $F_{1,5} = 5.495$, p = 0.066).

The contrasting effects of *Eda* on growth in saltwater and freshwater are primarily the result of effects occurring relatively late in development. We repeated our analysis on growth rate during two separate stages of development: juvenile and adult. We define juvenile growth as growth occurring from birth to the time that fish acquired their full adult number of lateral plates (Bell 1981, 2001; Barrett et al. 2008) (mean standard length = 27 mm). Adult growth is that occurring between the end of the juvenile stage and the end of the experiment (mean standard length = 44 mm). We found that the mean slope coefficients were similar in freshwater and saltwater treatments during juvenile growth (Figure 4.1B, linear mixed effects model on block slope coefficients, salinity: $F_{1,5} = 0.156$, p = 0.709). Juvenile growth rate was positively associated with the number of low alleles at the *Eda* gene in freshwater (mean of block slope coefficients = 0.0033, SE =

0.0015) as well as in saltwater (mean of block slope coefficients = 0.0028, SE = 0.0009). In contrast, there was a significant difference in the effect of *Eda* on adult growth between the treatments (Figure 4.1C, linear mixed effects model on block slope coefficients, salinity: $F_{1,5} = 12.675$, p = 0.016). In freshwater the relationship between growth and number of low alleles was positive (mean of block slope coefficients = 0.0017, SE = 0.0024) whereas it was negative in saltwater (mean of block slope coefficients = -0.0068, SE = 0.0023). Therefore, in saltwater growth of homozygous complete and heterozygous genotypes caught up to that of the homozygous low genotypes, whereas in freshwater the disadvantage of possessing complete alleles persisted through adulthood. This change in the pattern of slopes, comparing juveniles (Figure 4.1B) with adults (Figure 4.1C), was significantly different between saltwater and freshwater treatments (linear mixed effects model on the change in block slope coefficients, salinity: $F_{1,5} = 13.43$, p = 0.015). Thus, *Eda* has different pleiotropic effects in freshwater and saltwater, but this difference is dependent on ontogeny. Overall, having more low alleles, which reduces number of lateral plates, resulted in faster growth in freshwater but not in saltwater (Figure 4.1A and C).

Discussion

Pleiotropy is thought to be one of the most common properties of genes (Dobzhansky and Holz 1943; Barton 1990; Otto 2004), but rarely have the pleiotropic effects of candidate genes been measured in different environments. Testing how pleiotropic effects interact with the environment will greatly facilitate our understanding of the role that genetic architecture can play in promoting or constraining phenotypic diversification. By tracking

genotyped individuals throughout life, we were able to identify whether the *Eda* locus has environment-specific pleiotropic effects on two fitness-related traits. Our results show that in freshwater stickleback experience a tradeoff between armor and growth, because fish carrying the complete allele have high armor but reduced growth rate. Given this pleiotropy, the predominance of low plated populations in freshwater may be due to the growth rate advantage of this allele rather than a disadvantage of armor as such. Depending on the relative strength of selection acting on growth and armor, the low allele may be favored even when armor is under positive selection. A tradeoff between armor and growth also exists in saltwater, but only during juvenile growth. In the absence of an adult size advantage of the low allele, it will be selected against in the ocean if disadvantages of reduced armor in adult sticklebacks outweigh the advantages of increased growth in juveniles. Thus, environment specific pleiotropic effects may cause the direction of selection on *Eda* to vary across environments, even if the direction of selection on both traits remains constant.

We ended the experiment when we observed fish entering reproductive condition because breeding substantially alters how much energy is devoted towards growth (Day and Taylor 1997). It is possible that if the experiment had been continued for longer, the completely plated fish would have eventually reached similar adult sizes to low plated fish in freshwater. Juvenile growth in saltwater was faster than in freshwater (Figure 4.1B) and low plated fish may have reached an upper size limit earlier, allowing completely plated fish to 'catch-up'. In contrast, in freshwater low plated fish may not have reached their upper size limit during the experiment and therefore maintained a growth rate advantage throughout the adult stage. Regardless of whether genotypes

would have eventually reached equivalent sizes in freshwater, size differences occurring through the developmental stages that we measured in this study would have important fitness consequences in wild fish through effects on overwinter mortality and breeding time (Schultz et al. 1991; Curry et al. 2005; Barrett et al. 2008).

This experiment used offspring of wild-caught individuals rather than near-isogenic experimental lines, which means we cannot rule out the possibility of effects on growth of genes linked to the *Eda* locus. If linked genes are responsible for differences in growth between *Eda* genotypes, then recombination could eventually eliminate the association between the low allele and increased growth rate. Indeed, mapping studies using crosses of individuals from distantly related populations have found no evidence that *Eda* explains any variation in body size (Colosimo et al. 2005; Albert et al. 2008; Marchinko 2009). Regardless of whether the association between armor and growth detected in this study is due to pleiotropic effects of *Eda* alone or the pleiotropic effects of a group of tightly linked genes including *Eda*, the functional result will be the same upon colonization of freshwater; the low allele will increase in frequency due to its associated growth advantage and this will lead to the evolution of reduced armor. However, the association between reduced armor and increased growth will break down over time if linkage is playing a more important role than pleiotropy. The strength of our approach is that it used naturally occurring standing genetic variation, which was presumably the source of variation available to selection when fish first colonized freshwater at the end of the last ice age.

Our results have implications for the genetics of speciation in stickleback because assortative mating between marine and stream stickleback populations is strongly

influenced by body size (Ishikawa and Mori 2000; McKinnon et al. 2004). Marine sticklebacks are consistently larger in size than stream sticklebacks (McKinnon et al. 2004) and size differences are heritable (McPhail 1977; Snyder and Dingle 1989). *Eda* may influence this size difference after colonization of freshwater by completely plated marine sticklebacks. We found that homozygous complete genotypes raised in freshwater were on average 2.6 mm smaller than homozygous complete genotypes raised in saltwater. This difference accounts for approximately 9% of the magnitude of difference typically observed between freshwater and marine populations, suggesting that the slower growth in freshwater caused by *Eda* could have a moderate influence on assortative mating between marine and newly-formed stream stickleback. In contrast, the eventual fixation of the low allele in freshwater, which has happened countless times in postglacial streams across the northern hemisphere (Colosimo et al. 2005), will have the opposite effect on assortative mating. In freshwater, the low allele will cause an increase in body size, which will diminish the size difference between marine and stream-resident fish. This effect could serve to increase gene flow between environments and may help to explain how the low allele has been maintained at low frequency in marine environments for over 2 million years (Colosimo et al. 2005). Understanding the overall pleiotropic effects of candidate genes in different environments can provide a more comprehensive view of the mechanisms that drive patterns of phenotypic evolution.

Table 4.1. Association between Eda genotype and armor phenotype

	Armor phenotype		
Eda genotype	Complete	Partial	Low
CC	98	5	0
CL	54	61	3
LL	2	43	47

Goodman and Kruskal $\Upsilon = 0.70$, $\chi^2 = 226.57$, d.f. = 4, $p < 10^{-15}$, n = 313. Individuals pooled from all blocks. There were no significant treatment effects on genotype-phenotype associations (heterogeneity $\chi^2 = 8.1$, d.f. = 4, p = 0.09). *Eda* genotypes are based on the Stn381 in/del marker: "complete" (C) alleles represent 162 or 171 bp bands, and "low" (L) alleles represent 191 bp bands.

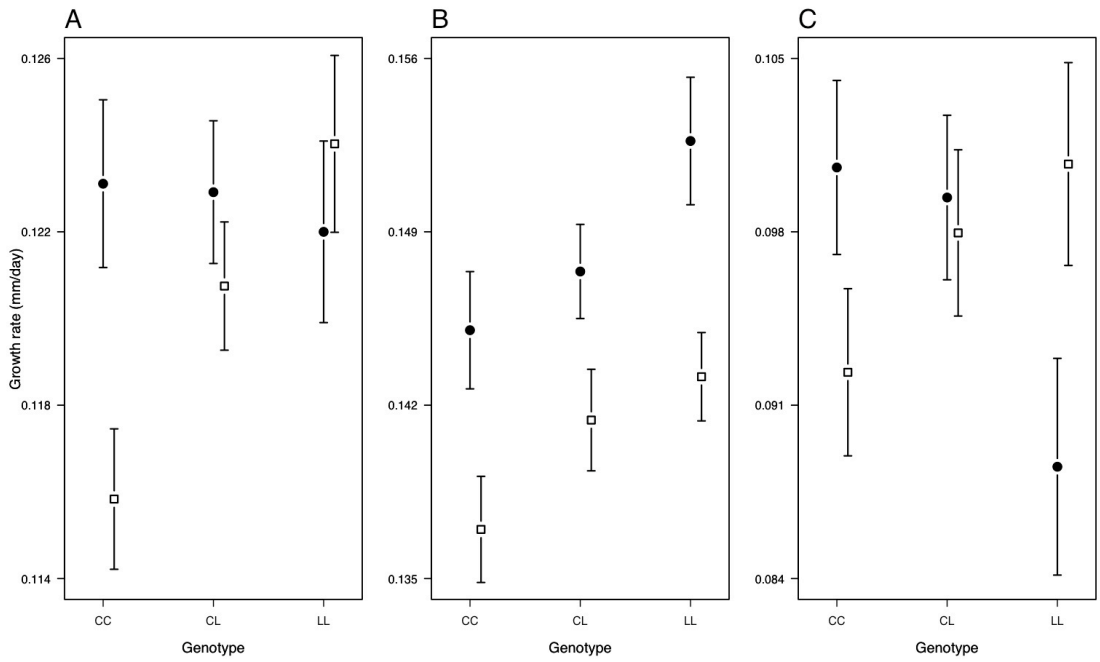

Figure 4.1. Growth rates of Eda genotypes in salt and fresh water.

(A) Total growth rate by *Eda* genotype in salt (●) and fresh water (□). (B) Juvenile growth rate by genotype, calculated from birth to the end of adult plate number development at average standard length of 27 mm. Symbols as in (A). (C) Adult growth rate by genotype, calculated between juvenile growth and the end of the experiment at average standard length = 44 mm. Symbols as in (A). Error bars show ± 1 standard error. Note different scale on vertical axis in each panel.

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5. SHOULD I STAY OR SHOULD I GO? THE ECTODYSPLASIN LOCUS IS ASSOCIATED WITH HABITAT PREFERENCE IN THREESPINE STICKLEBACK [4]

Introduction

Adaptive divergence is hampered when gene flow from the ancestral range introduces locally deleterious alleles and thus impedes local adaptation (Kawecki & Ebert 2004). This effect may be mitigated if alleles are more likely to move to environments where they have high fitness (Jaenike & Holt 1991). This 'matching habitat choice' (Edelaar et al. 2008) can arise when locally adaptive loci have pleiotropic effects on habitat preference, or when they are tightly linked to loci that confer preference. There is evidence that pleiotropic effects are common for candidate genes affecting behaviour, but examples are still limited to just a few systems (Fitzpatrick et al. 2005, Sokolowski 2001).

The gene *Ectodysplasin* (*Eda*) is largely responsible for variation in defensive armor (bony lateral plates) in threespine stickleback *Gasterosteus aculeatus* populations (Colosimo et al. 2005). Fish homozygous for 'complete' alleles are common in the ocean and typically possess a row of 30 to 36 plates (complete morph), whereas homozygotes for 'low' alleles are common in freshwater and typically possess 0 to 9 plates (low morph) (Bell & Foster 1994; Hagen & Gilbertson 1972). Heterozygotes are rare in both environments and possess an intermediate number of plates (partial morph) (Bell & Foster 1994; Hagen & Gilbertson 1972). Lateral plates play a defensive role in stickleback, increasing the difficulty of ingestion by predatory vertebrates (Reimchen

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1983) and also the probability of escape and survival after capture (Reimchen 1992; Reimchen 2000). The complete allele is probably favoured in oceanic habitats because sticklebacks are often far from cover and experience intense vertebrate predation pressure (Bell 2001; Colosimo et al. 2005; Marchinko 2008; Reimchen 2000). In contrast, the low allele is favoured in freshwater due to beneficial effects on growth rate that lead to higher overwinter survival (Barrett et al. 2008; Marchinko & Schluter 2007). The low allele originated more than two million years ago (Colosimo et al. 2005), but the postglacial lakes commonly inhabited by low-armor freshwater populations have only existed for ~10,000 years, implying that evolution of the low-armor phenotype has occurred by recurrent local selection on an ancient allele brought repeatedly into freshwater environments by marine founders.

Here, we use a laboratory experiment with the F1 progeny of wild marine sticklebacks heterozygous at the *Eda* locus to test if there are significant differences in behaviour between genotypes. Specifically, we test if genetic variation at *Eda* is associated with preference for freshwater versus saltwater after acclimation to each salinity. Adaptive divergence in stickleback armour morphology would be facilitated if the low allele conferred a consistent preference for freshwater environments.

Methods

Sample populations

We collected marine sticklebacks in April and May of 2006 from Oyster Lagoon on the Sechelt peninsula in western British Columbia (49°36'48.6" N, 124°1'46.88" W). Oyster Lagoon is a saltwater inlet with salinity ranging from 28-32 ppt, in which phenotypically

partially plated fish occur at an approximate frequency of 0.01. This population breeds in saltwater and the rare sticklebacks with reduced plate number are marine in all other phenotypic traits (shape, size, color, spine length; Foster 1994; Siamoto 1995; Barrett et al. 2008). Fish with reduced armor predominantly (96% of *Eda* heterozygotes; Barrett et al. 2008) carry alleles consistent with marine residency at a SNP within an α1 subunit of Na+-K+-ATPase (Jones et al. 2006). We sampled approximately 10,000 fish using unbaited minnow traps. We genotyped partially plated fish to confirm that they were *Eda* heterozygotes (see below). Using artificial fertilization, we generated families from these heterozygotes and raised offspring in 102L freshwater (0 ppt) aquaria on a diet of brine shrimp during juvenile growth and bloodworms during adult growth. Fish were kept at 17º C and 16h:8h light:dark regime, and were an average of 44.0 mm (± 5.0 SD) in length and 18 months old at the start of the experiment.

Genotyping

We genotyped fish at diagnostic sites within the *Eda* gene that distinguish between low and complete morph alleles. We isolated total genomic DNA from small caudal fin clips using a standard proteinase K phenol chloroform protocol (Sambrook et al. 1989). We used a diagnostic indel locus, Stn381, to identify low and complete *Eda* alleles (Colosimo et al. 2005). *Eda* alleles were amplified by PCR using a DNA Engine® Peltier Thermal Cycler (MJ research, Inc.) in 10 ul reactions containing 5 to 15 ng of genomic DNA, 1uM of each forward and reverse primer, 1X PCR buffer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.25U of AmpliTaq Gold polymerase (Applied Biosystems). Cycling conditions were standardized over all loci as follows: 93 ^{o}C for 3 min, 95 ^{o}C 30 s, 59 ^{o}C

30 s, 72 °C 30 s, 5 cycles of 94 °C 30 s, 59 °C 30 s, 72 °C 30 s, 35 cycles of 90 °C 30 s, 60 °C 30 s, 72 °C 30 s, followed by 72 °C for 10 min and then cooled to 4 °C. Electrophoresis consisted of pooling PCR products with an internal size standard (LIZ 500bp, Applied Biosystems) and loading onto the Applied Biosystems 3730S Automated Sequencer. Allelic sizes (in base pairs) were determined by reference to the internal sizing standard in the software GENEMAPPER (Applied Biosystems).

Preference experiment

We conducted the preference experiment in 21 L (40x20x15 cm) aquaria, each containing a plexiglass sheet 3 cm shorter than the height of the aquaria. We randomly selected one side of each aquarium to fill with artificial saltwater (30 ppt, 17º C; Instant Ocean synthetic sea salt, Aquarium Systems, Inc., Mentor, OH) up to the level of the dividing sheet. We then filled the other side of the aquarium with freshwater (0 ppt, 17º C) up to the top of the aquarium, creating a 2 cm freshwater bridge between the two sides. We placed size matched non-experimental fish in jars with a mesh top on either side to promote normal schooling behaviour (Barber & Ruxton 2000) (Figure 5.1). To initiate the preference experiment we introduced a freshwater-acclimated test fish into the saltwater side of the aquarium. After a five-hour settling period, we recorded fish location every 15 minutes for 4 hours using a QuickCam Pro 9000 by Logitech (Fremont, California) webcam and Evocam software (http://www.evological.com). Data from fish that did not visit both sides prior to the data collection period (n=2) were discarded. We tested all fish within 24 days. We then acclimated fish to saltwater for between 35 and 50 days under the same laboratory conditions. Stickleback plasma osmolarity, which is a

strong indicator of osmotic condition, takes 7 days to stabilize following transfer from freshwater to saltwater, so this acclimation period is more than sufficient to allow osmotic acclimation to saltwater (Schaarschmidt et al. 1999). We then repeated the preference experiment using the protocol above, except that fish were first introduced to the freshwater side of the aquaria. All fish were tested in the second trial within 28 days. We introduced fish to the unacclimated salinity in each trial so that they would initially experience osmotically stressful conditions and be encouraged to sample both environments. We tested 81 fish in the freshwater acclimation trial (30 homozygous complete, 28 heterozygotes and 23 homozygous low), and 75 in the saltwater trial (29 homozygous complete, 25 heterozygotes and 21 homozygous low).

We determined preference for freshwater as the proportion of time spent by a fish in freshwater during a trial. We calculated this by scoring location as 1 if the fish was observed in freshwater and 0 if observed in saltwater and then averaging over all observation periods. Similarly, we determined preference for acclimation environment by scoring location as 1 if observed in the acclimation environment and 0 if observed in the alternate environment and then averaging over all observation periods. To determine the influence of *Eda* genotype on preference, we employed a linear mixed effects model in R 2.7.0 (R Development Team 2008) to test for an association between the number of low alleles possessed by an individual (0 for homozygous complete, 1 for heterozygote, 2 for homozygous low) and preference score. Genotype and environment (saltwater versus freshwater or acclimated versus unacclimated) were treated as fixed effects and individuals were treated as random effects. To account for the possibility that differences in preference may be influenced by differences in activity level between genotypes, we

also scored the number of times a fish had moved between environments and included this term as a fixed effects covariate in our model.

Results

We found no significant difference between genotypes in preference for freshwater, but we found a strong interaction between genotype and acclimation environment (linear mixed effects model on preference for freshwater, genotype: $F_{1,80} = 0.231$, p = 0.632; genotype by acclimation environment: $F_{1,66} = 8.104$, p = 0.006). This interaction indicates that homozygous complete genotypes spend a greater proportion of time in the acclimation environment than homozygous low and heterozygous genotypes. If the data are reanalyzed using a linear mixed effects model on preference for acclimation environment and genotype, we find strong support for an effect of *Eda* genotype (Figure 5.2; $F_{1,80} = 7.368$, p = 0.008). We found no evidence for differences in movement rate between genotypes (genotype by movement: $F_{1,66} = 0.407$, p = 0.526).

Discussion

We found the first evidence of a locus associated with behaviour in threespine stickleback. Our results provide no support for an association between the low allele and preference for freshwater. Instead, fish carrying a low allele at the *Eda* locus showed a preference for the alternative environment over the acclimation environment. The main behavioural difference between *Eda* genotypes is thus that fish carrying the complete allele prefer to stay in the salinity to which they have been acclimated, whereas fish carrying the low allele prefer to move to different salinities. The mechanistic basis for

this observation is unknown but could be due either to direct effects of the *Eda* locus or the effects of closely linked genes. Mutations in both *Eda* and the *Eda* Receptor (*Edar*) have been shown to affect the number and structure of gill rakers in zebrafish (Harris et al. 2008), opening the possibility of effects on the functional properties of gills. Alternatively, *Eda* is tightly linked to vacuolar proton translocating ATPase subunit a isoform 3 and sodium/hydrogen exchanger 6, two proteins known to be important in ion uptake mechanisms of freshwater fish (Evans et al 2005), and *Eda* could be acting as a marker for variation at these loci. Thus, it is possible that the low allele is associated with physiological changes that allow increased tolerance of altered ionic conditions. However, it is unclear why this would lead to a preference for these conditions over the acclimation conditions.

The differences in behaviour observed between *Eda* genotypes in this study may have implications for the long-term maintenance of variation at this locus in natural populations. Our results suggest that marine sticklebacks carrying the low allele may be more likely to colonize freshwater environments, where positive selection can then act to increase its frequency (Barrett et al. 2008; Colosimo et al. 2005). However, freshwater acclimated sticklebacks carrying the low allele will be more likely to leave freshwater for the ocean. This trait may impede local adaptation by promoting migration of low alleles between environments, and could help to explain the persistence of the allele at low frequencies in the ocean despite presumably negative selection in this environment (Colosimo et al. 2005). Experimental tests of the behavioural effects of genes under divergent selection allow for a more comprehensive understanding of the mechanisms leading to local adaptation and the maintenance of genetic variation.

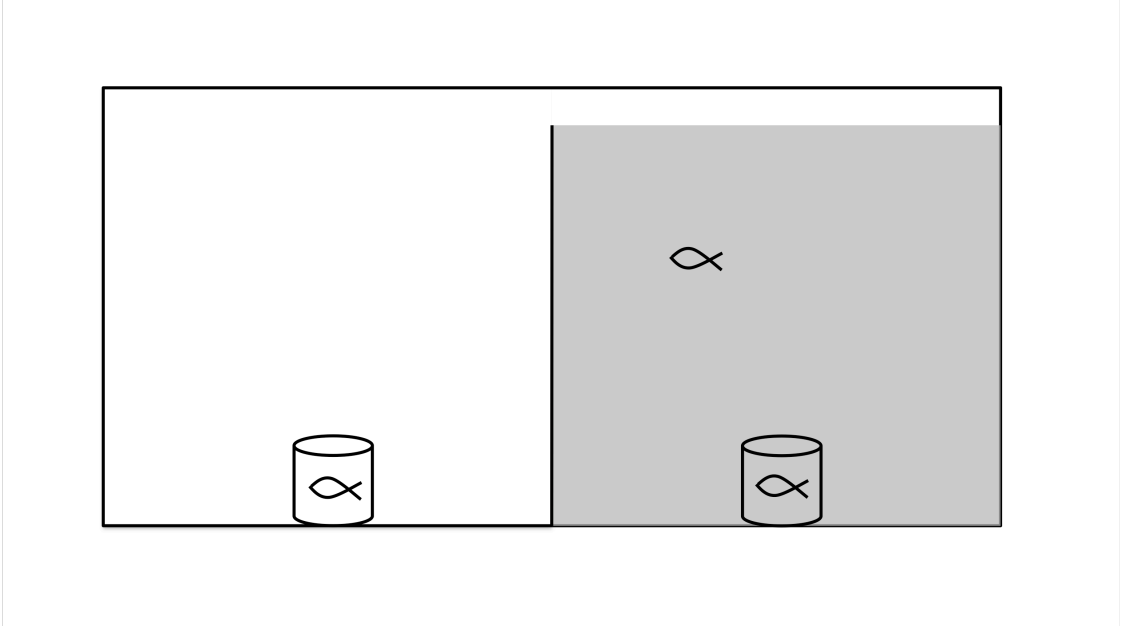

Figure 5.1. Schematic of the experimental design for preference trials.

White represents freshwater (0 ppt) and grey represents saltwater (30 ppt). See methods.

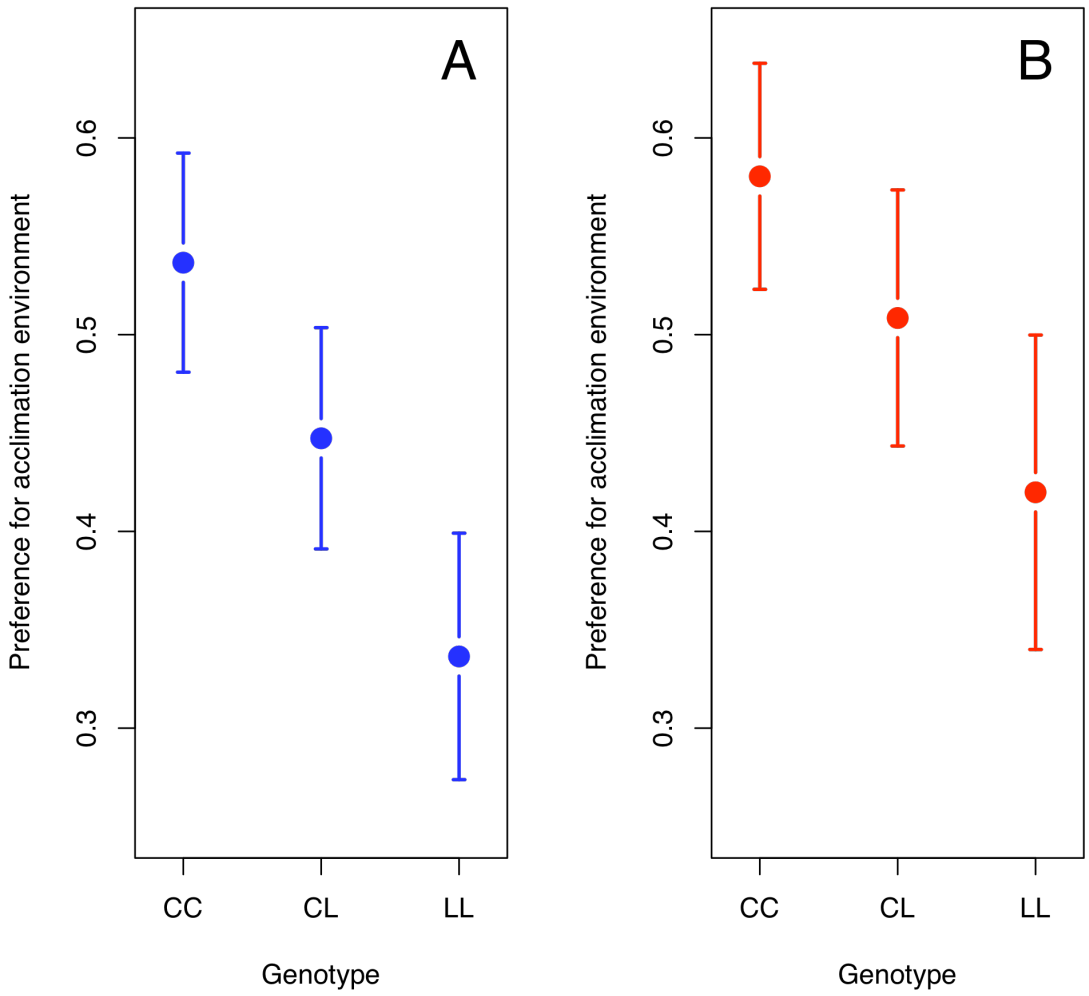

Figure 5.2. Preference for acclimation environment after acclimation to freshwater (A) and saltwater (B).

Preference represents the average preference score for all individuals of each *Eda* genotype. A preference of 1 indicates complete preference for the acclimation environment and 0 indicates complete preference for the alternate environment. Error bars represent ±1 standard error.

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6. ADAPTIVE EVOLUTION OF LATERAL PLATES IN THREESPINE STICKLEBACK: A CASE STUDY IN FUNCTIONAL ANALYSIS OF NATURAL VARIATION[5]

Introduction

With close to 30,000 species covering a bewildering array of body forms and ecological roles, fishes represent a spectacular example of global biodiversity. Understanding the mechanisms responsible for the creation and maintenance of this diversity is a central goal of biological research, and one that requires the synthesis of knowledge from evolution, ecology, and molecular biology. Precise fit between form and ecological function suggests the influence of adaptive evolution, but actually testing hypotheses about the role of natural selection in phenotypic differences has proven difficult. Historically, two parallel approaches have been used to study adaptive evolution, with population biologists focusing on how individuals differ in phenotypic traits across environments, and population geneticists investigating spatial and temporal changes in allele frequencies. These approaches are complementary, because although selection acts on phenotypes regardless of their genetic basis, the evolutionary response to selection is determined by the underlying genetic architecture of these traits. With the emergence of powerful genomic techniques, it is possible to combine these approaches by linking molecular changes to the phenotypes responsible for adaptive differences between populations (i.e., finding "the genes that matter"). However, while making the connection between genotype and phenotype is a laudable accomplishment, it cannot in itself identify the functional mechanisms or ecological context by which selection favours one allele

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over another and thereby creates phenotypic differences between populations. A powerful approach to solving this problem is to conduct field experiments with selected genotypes to evaluate directly the fitness consequences arising from the phenotypic effects of specific alleles (Bradshaw and Schemske, 2003; Lexer *et al.*, 2003; Baack *et al.*, 2008; Barrett *et al.*, 2008; Wegner *et al.*, 2008). This approach would represent a step toward the synthesis of genomics and population biology that should provide estimates of the timing and strength of selection, enhance understanding of the genetics of adaptation, and yield insights into the mechanisms driving changes in gene frequency.

Progress towards the functional analysis of genetic variation in fishes is now being driven by research using model species with excellent genomic resources (Cossins and Crawford, 2005). For instance, comprehensive genomic tools have been developed in several Actinopterygian groups: stickleback, medaka, fugu, zebrafish and cichlids. These include full genome sequences, genetic maps, gene expression techniques, transgenic techniques, reverse genetic tools, forward genetic screens, express sequence tag (EST) databases, and bacterial artificial chromosome (BAC) libraries (Abzhanov *et al.*, 2008). Furthermore, genomic resources for functional analyses are rapidly being developed and applied in several other groups of fishes, such as killifish (Oleksiak *et al.*, 2005; Fangue *et al.*, 2006) and salmonids (e.g., lake whitefish *Coregonus clupeaformis* (Mitchill) and Atlantic salmon *Salmo salar* (L.)) (Waples and Naish, 2008; Bernatchez *et al.*, 2009). Natural characteristics of these groups such as the presence of readily identifiable ecotypes and the viability of crosses from divergent populations add to their usefulness for investigating adaptive genetic differences in the wild. In the case of stickleback and cichlids, decades of ecological and evolutionary study have preceded their more recent

role as molecular model systems and provide a valuable real-world context within which to interpret genomic data.

Of all the taxa mentioned above, stickleback may provide the best combination of diverse ecological variation, interesting evolutionary history and well-developed genomic resources. Three-spined stickleback *Gasterosteus aculeatus* L. is a small (<10cm SL) holarctic fish with marine, anadromous, and freshwater populations (Bell and Foster, 1994). The species can be distinguished by bony armour and spines, bright male nuptial colouration, and elaborate courtship behaviour. Relative to marine stickleback, the stickleback inhabiting postglacial freshwater habitats have evolved a wide diversity of armour, colour, diet, trophic morphology, body forms, behaviour, and life history traits. Some of this variation, such as presence or absence of a pelvis, rivals interspecies differences in other groups (Bell, 1987; Cresko *et al.*, 2004). Furthermore, the evolutionary history of these fish provides an exceptional opportunity to study the genetic architecture of adaptive divergence. Genetic evidence shows that marine stickleback are ancestral to many freshwater populations, which have repeatedly colonized lakes and streams created after the retreat of the Pleistocene glaciers (Hagen and McPhail, 1970; Rafinski *et al.*, 1989; McPhail, 1994; Taylor and McPhail, 1999; Reusch *et al.*, 2001; Raeymaekers *et al.*, 2005). Thus, the profound morphological, behavioural and physiological differences between marine and freshwater populations in recently deglaciated regions have evolved in a remarkably short period of time, presumably in response to local ecological conditions (McKinnon and Rundle, 2002). In this short review several decades of research investigating the adaptive divergence of the lateral plate armour phenotype in three-spined stickleback are outlined. This topic has been

reviewed previously (Reimchen, 1994; Bell, 2001), and so a focus is placed on recent efforts to experimentally measure selection on the genes that underlie this trait. This work will hopefully serve as an example of how connecting genotype to phenotype to fitness allows a more comprehensive understanding of the mechanisms that create and maintain biological variation in fishes.

Phenotypic variation in lateral plate number

One of the most characteristic morphological differences between marine and freshwater stickleback is the reduced number of bony lateral plates in many freshwater populations (Bertin, 1925; Heuts, 1947). Lateral plates are superficial dermal bones that form a single row numbering between 0 and 36 along each side of the body (Scott and Crossman, 1973). There is quantitative variation both within and among populations. Marine stickleback typically have a continuous row of over 30 plates spanning from head to tail (complete morph) (Figure 6.1). In contrast, freshwater stickleback typically possess fewer than 10 plates, usually at the anterior end of the body (low morph). A small proportion of both marine and freshwater fish have intermediate numbers of plates, most often with plates absent from the middle of the body (partial morph). The sequence of plate development proceeds with anterior plates developing first, followed by posterior plates, and finally middle plates (Bell, 2001).

Consistent changes in the frequency of lateral plate morphs have been observed during contemporary evolution following colonization of freshwater by marine stickleback. Significant reductions in lateral plate number have been documented in recently founded freshwater populations in Norway, Iceland and Alaska (Klepaker, 1993;

Kristjansson *et al.*, 2002; Bell *et al.*, 2004; Kristjansson, 2005). These changes have occurred progressively over several generations during time periods of 40 years or less, consistent with evidence that lateral plate phenotype is heritable and subject to selection (Bell, 2001). A number of hypotheses have been proposed as potential mechanisms driving the evolution of reduced armour in freshwater environments. These hypotheses can loosely be grouped into those involving either biotic or abiotic mechanisms. Below I outline the work that has tested each.

Biotic mechanisms

It has frequently been suggested that lateral plate morphology is under divergent selection across environments because of differing predation risks (Hagen and Gilbertson, 1973; Moodie *et al.*, 1973; Bell and Haglund, 1978; Reimchen, 1983; 1991; 1992; Bell and Foster, 1994; Reimchen, 2000; Bell, 2001; Vamosi, 2002; Kitano *et al.*, 2008; Marchinko, 2009). Marine and lake habitats with a high abundance of large fish usually have stickleback populations composed of the complete morph (Reimchen, 1994). Increased piscivore predation intensity has been associated with positive selection on lateral plate number during contemporary evolution in Lake Washington, USA (Kitano *et al.*, 2008). Lateral plates serve a defensive role against predatory fish. Anterior plates connect dorsal and pelvic spines, mechanically bracing the structures against each other. This allows a stickleback to maintain erect spines, which creates the mechanical integrity required to pierce the mouth of piscivorous vertebrates and also provides a greater effective diameter, which increases the difficulty of ingestion by gape-limited predators (Reimchen, 1983). More obviously, plates can act as a protective barrier against puncture

injury from toothed predators. Experimental evidence shows that stickleback with greater numbers of anterior plates are more likely to survive after capture by predatory fish (Reimchen, 1992), helping to contribute to the high (90%) failure rate of these attacks (Reimchen, 1991). Greater overall numbers of plates can also obstruct swallowing by piscivorous fishes, most likely by hindering pharyngeal jaw retraction. The increased difficulty of swallowing leads to greater handling time and more escape opportunities (Reimchen, 2000). Cumulatively, these various biomechanical advantages of lateral plates are thought to result in positive selection on lateral plate number in environments where stickleback are regularly captured by predatory fish.

In contrast to the open water habitat of many marine environments, freshwater environments possess greater proportions of littoral habitat where stickleback can presumably be closer to cover (Reimchen, 1994). Under these conditions, stickleback may be more likely to successfully evade attacks and the importance of evasion may supersede the importance of escape and survival after capture (Reimchen, 2000; Bell, 2001). Greater numbers of plates are associated with reduced body flexure and velocity during fast-start escapes, suggesting that completely plated stickleback may be easier to catch (Taylor and McPhail, 1986; Bergstrom, 2002). This may be especially relevant in cases of significant avian predation because diving birds, which only submerge for brief periods and hold stickleback with compression rather than puncturing, decrease the protective advantage of plates compared to burst speeds required to escape (Reimchen, 1994). Furthermore, in some freshwater habitats aquatic invertebrate predation may also select for reduced armour. Aquatic insects prey on juvenile stickleback (Foster *et al.*, 1988) and it has been hypothesized that armour serves as a point of leverage for the

predators to grasp during capture (Reimchen, 1980). Thus, a reduction in lateral plate number may be advantageous in freshwater habitats because it improves the likelihood of avoiding predation entirely.

Abiotic mechanisms

Abiotic differences between marine and freshwater environments have long been considered as possible mechanisms causing diversification in armour morphology (Heuts, 1947). Teleost fish absorb calcium from ambient water for skeletal development (Peterson and Martin-Robichaud, 1986; Marshall, 2002; Evans *et al.*, 2005). Reduced concentrations of calcium, phosphate, and other ions in many freshwater environments relative to the ocean are hypothesized to make it increasingly costly to mineralize the skeletal components required to build lateral plates (Giles, 1983). Under this hypothesis, possessing fewer plates would be advantageous in low ion concentration freshwater lakes and streams but not necessarily disadvantageous in high ion concentrations. Correlations between ion concentration and amount of skeletal armour across freshwater environments provide evidence that is consistent with ion limitation leading to reduced numbers of lateral plates (Giles, 1983; Bell *et al.*, 1993; Bourgeois *et al.*, 1994; Schluter, 1995). It should be noted that ion limitation may also have an indirect effect on lateral plate number in freshwater by limiting the distribution of predatory fish, which cannot tolerate the low pH associated with calcium limited lakes (Muniz, 1991). Ion limitation and reduced predation are therefore not mutually exclusive hypotheses for the evolution of reduced armour (Bell *et al.*, 1993).

An additional hypothesis involving abiotic differences between marine and freshwater environments is that there is a trade-off between salinity tolerance and lateral plate number. An early experiment investigating this idea found that complete plate morphs survive longer and hatch more successfully in high salinities, whereas low plate morphs survive longer and hatch more successfully in low salinities (Heuts, 1947). While suggestive, the plate morphs used in this study were collected from different environments and thus there may be other local adaptations contributing to the performance of the morphs in addition to lateral plates. A more recent study avoided this problem by testing the salinity tolerance of plate morphs collected from the same populations. The results supported earlier findings of reduced growth rate of complete plate morphs in freshwater environments, but did not find evidence of a difference in growth in saltwater or differences in hatching success in either ionic strength (Marchinko and Schluter, 2007). Together, this work suggests that positive selection for low plate morphology may be the result of a correlated response to selection for increased growth rate under low salinity conditions. Whether the predominance of the low plate morph in freshwater is due to differences in salinity tolerance between morphs or the difficulty of building plates under ion limitation, a key point is that the mechanism driving evolution of plate morphology could differ between environments (Marchinko and Schluter, 2007). In freshwater habitats, functional constraints imposed by abiotic properties of the environment may result in the evolution of reduced armour, whereas in the ocean, complete armour may persist due to selection from high intensity fish predation.

Genetics of lateral plate phenotype

The genetics of lateral plates in stickleback have been intensively studied for decades (reviewed in Banbura and Bakker, 1995; Bell, 2001). Here I focus on recent studies that have greatly advanced understanding of this topic through the use of genome wide linkage mapping approaches to identify the specific molecular changes responsible for lateral plate differences. Despite the diversity of life history, morphological and behavioural traits in stickleback, viable crosses can be produced from almost any two populations from around the world, greatly facilitating the use of genetic mapping (Peichel, 2005). This approach obviates the need for a more traditional strategy using candidate genes. By definition, a candidate gene approach is limited to looking for variation in already known genes that have been conserved across widely divergent taxa. These genes have typically been chosen based on mechanistic knowledge about the links between a phenotypic trait and a candidate locus (Hoffmann and Willi, 2008). In contrast, genetic mapping makes no prior assumptions about the genes involved in a phenotypic trait and is therefore capable of finding previously unknown loci.

To identify genes responsible for variation in lateral plates, genotype-phenotype associations were investigated in the F2 offspring from a divergent cross. A complete plate morph from the Japanese Sea was crossed with a low plate morph from Paxton Lake in British Columbia (Figure 6.2A). The resulting F1 progeny were then crossed to produce an F2 generation that segregated variation in lateral plate number and a number of other traits that distinguish the two grandparents. These F2 individuals were genotyped with 428 microsatellite markers spanning the three-spined stickleback genome (Colosimo *et al.*, 2005) (Figure 6.2B). The markers identified six loci that interact semi-additively to

determine lateral plate number. One of these loci, located on linkage group 4, was associated with a QTL controlling 80% of the variation in plate number (Colosimo *et al.*, 2004). To identify the specific gene underlying this QTL, genetic mapping was used to discriminate a 0.68 cM interval and bacterial artificial chromosome (BAC) clones covering this interval were determined (Colosimo *et al.*, 2005). Two BACs that covered the majority (400 kb) of the interval were then sequenced, which helped to identify a number of genes in this region. The interval was further narrowed with linkage disequilibrium mapping, leaving only a 16 kb region containing a few genes likely to be responsible for plate variation (Figure 6.2C). Among these genes is the secreted signalling molecule *Ectodysplasin* (*Eda*), a member of the tumour necrosis family. *Eda* was originally identified by its role in hypohidrotic ectodermal dysplasia (HED) in humans, which results in defects in the development of multiple ectoderm-derived structures, including hair, teeth, and sweat glands (Mikkola and Thesleff, 2003). HED patients also show characteristic changes in flat ectodermal bones of the skull, showing that EDA is required for normal development of some skeletal structures. In addition, mutations affecting the *Eda* receptor (*Edar*) impede formation of scales in zebrafish (Harris *et al.*, 2008) and medaka (Kondo *et al.*, 2001), which share a developmental origin with the dermal bone used in stickleback lateral plates. The importance of the *Ectodysplasin* signalling pathway for development of dermal bone and scales makes *Eda* a good candidate for controlling lateral plate number, but in order to conclusively show that the gene is causally responsible, transgenic techniques were used to test whether plate development could be altered by changing levels of *Eda* signalling (Colosimo *et al.*, 2005). Embryos from low plated stickleback parents were injected with an *Eda* cDNA

construct that is known to restore development of teeth, hair, and sweat glands when introduced into mutant mice carrying a null mutation at the *Eda* locus. Introduction of this construct resulted in higher levels of *Eda* expression and the development of extra plates in a small number of fish, suggesting that *Eda* transgenes are sufficient to trigger development of lateral plates (Figure 6.2D). However, despite identification of a strong association between *Eda* and lateral plates, the causative regulatory mechanism still remains unknown (Knecht *et al.*, 2007).

An exciting new development for high-throughput fine-scale genetic mapping of lateral plate phenotype is the use of restriction-site associated DNA (RAD) tags (Baird et al 2008). These markers are short fragments of DNA adjacent to each instance of a particular restriction enzyme recognition site. When RAD tag libraries are combined with next-generation sequencers it is possible to rapidly discover and map thousands of SNPs at low cost. RAD marker density is an order of magnitude greater than existing microsatellite marker density, which facilitates both fine mapping of previously known genetic regions associated with a particular phenotype, and also the discovery of additional unknown regions. This approach has recently been used to discover regions associated with lateral plate phenotype that share the same linkage group but are 7 to 12 Mb physically distinct from *Eda* (Miller *et al.*, 2007; Baird *et al.*, 2008). Importantly, when reanalyzed without taking advantage of any available reference genome information, tags linked to lateral plate phenotype were again found in the same three regions, suggesting that sequencing of RAD tags would provide useful markers even in fish that do not have a reference genome.

Selection on Eda and the mechanisms responsible

Identifying differences in the ecological performance of phenotypic variants and the genes that underlie them are the first steps in the functional synthesis of evolutionary biology (Arnold, 1983; Kingsolver and Huey, 2003; Dalziel *et al.*, 2009). The final element is to quantify natural selection acting on the genes to shed light on the functional mechanisms and evolutionary forces that have shaped patterns of variation. Although they cannot provide an estimate of the strength of selection, instances of parallel evolution give strong evidence of past selection. Natural selection has almost certainly played a role whenever the same genetic changes and phenotypic traits evolve repeatedly and consistently in association with the environment (Simpson, 1953; Endler, 1986; Schluter and Nagel, 1995; Schluter *et al.*, 2004). Parallel evolution could also occur through genetic drift, but it is highly unlikely that transitions would be consistently associated with the environment. Genetic mapping and complementation crosses show that *Eda* is responsible for the repeated evolution of the low plate morph in freshwater throughout the world (Avise, 1976; Colosimo *et al.*, 2004; Cresko *et al.*, 2004; Schluter *et al.*, 2004; Colosimo *et al.*, 2005). Almost all low plated freshwater populations examined share a number of nucleotide changes within and near the *Eda* gene, while a different set of related haplotypes are shared by completely plated marine populations. Phylogenetic analysis of these sequences grouped almost all populations according to their plate morph. This pattern, and the discrepancy with the topology of the tree obtained with 25 random neutral markers, which grouped populations by geography rather than plate phenotype, strongly suggests the low plate haplotype is favoured in freshwater because

genetic drift is unlikely to create such a strong correlation between genotype and environment.

A few recent studies have explicitly tested whether freshwater populations have experienced selection at the *Eda* locus by analyzing patterns of sequence polymorphism at and around the gene in comparison to neutral markers in other regions of the genome. Separating the effects of selection from demography can complicate detection of this signal since population expansions or bottlenecks can result in similar patterns to the sweep of a beneficial allele (Slatkin and Wiehe, 1998; Nielsen, 2001; Kim and Stephan, 2002; Santiago and Caballero, 2005). However, the prediction is that if *Eda* mutations resulting in the low plate morph are under strong positive selection in freshwater there should be a reduction in polymorphism that is restricted to the neighbouring region, whereas demographic effects would leave a genome-wide signal. This is because the low plate alleles will occur with only a subset of neutral variants at linked sites, creating linkage disequilibrium between them. Although mutation and recombination can obscure this signature, the fixation of low plate alleles is expected to be recent enough (10-15,000 years) to often leave a distinguishing "signature of selection" in the surrounding genomic region. In agreement with this prediction, a genome scan of 103 microsatellite and two indel markers in four freshwater and three marine Fennoscandian populations found that the strongest signal of directional selection emerged from markers within the intronic regions of *Eda* (Makinen *et al.*, 2008). Similarly, F_{ST} values at markers within the *Eda* gene were significantly lower than F_{ST} values at neutral markers in an analysis of six Belgian freshwater populations (Raeymaekers *et al.*, 2007). In contrast, when a marker more distantly linked to *Eda* was analyzed in marine and freshwater populations, no

allele frequency differentiation was found despite greater differentiation in plate number than expected by genetic drift ($Q_{ST} > F_{ST}$; Cano *et al.*, 2006). These studies demonstrate that the major locus controlling variation in lateral plates does experience positive selection upon colonization of freshwater and therefore provide indirect evidence supporting an adaptive role for reduced armour in this environment.

Although identifying a molecular signature of selection can provide supporting evidence that a gene contributes to production of an adaptive phenotype, it cannot reveal the functional mechanism by which selection is acting on the gene. To address this, studies are now beginning to incorporate our growing knowledge of the genes underlying putatively adaptive traits with experiments to directly test hypotheses about the fitness consequences of functional differences between genotypes under natural conditions. This approach has recently been used to clarify the mechanisms driving evolution of reduced armour in freshwater stickleback. Marchinko (2009) tested whether insect predation would favour reduced armour by measuring selection in juvenile F2 families from crosses between freshwater (low plated) and marine (completely plated) populations. Families were split between control and insect predation treatments in experimental ponds. Predation was greatest on individuals carrying the complete allele (Figure 6.3A), providing support for the hypothesis that selection from aquatic insect predation contributes to the evolution of reduced armour in freshwater stickleback. In conjunction with the evidence for positive selection on armour when fish predation is high (Reimchen, 1992; 2000), these results indicate that divergent selection driven by differences in predation regime is one mechanism contributing to armour polymorphism across environments.

In two complimentary studies, Barrett and colleagues (2008; 2009) tested the hypothesis that when marine stickleback invade freshwater environments natural selection favours the low allele because it confers an advantage in growth rate. Under this hypothesis, the evolution of low plated populations in freshwater environments may be the result of a correlated response to positive selection for increased growth rate, rather than negative selection on armour (Marchinko and Schluter 2007). Barrett et al. (2008) tracked adaptive evolution over a complete generation at the *Eda* locus in replicated transplants of marine stickleback to freshwater environments. The experiment was designed to test if the *Eda* low allele is positively selected in freshwater because it permits a re-allocation of energy from lateral plates to growth rate. Increased growth rate can increase lipid stores and result in higher over-winter survival, a key component of fitness (Schultz *et al.*, 1991; Curry *et al.*, 2005). In order to control for the effects of background genetic variation, the study used only rare marine stickleback that were heterozygous at *Eda*. These fish carry the same genetic variation that selection acts on in nature and since the variation at *Eda* is imbedded within a marine genetic background, they can be used to help isolate the effects of selection on the locus from many of the other genetic differences between marine and freshwater environments. Over 35,000 fish were sampled from the ocean in order to obtain 182 heterozygotes, which were used to produce an F1 generation with all three *Eda* genotypes. In agreement with predictions, the low allele was associated with higher juvenile growth and improved overwinter survival, with a selection coefficient of ~0.5 against the complete allele over the winter months (Figure 6.4). Barrett et al. (2009a) used laboratory rearing experiments to test if *Eda*'s effects on growth rate are environmentally determined. If pleiotropy between

armour and growth is only present in freshwater this would facilitate divergent selection on *Eda* even with parallel selection on armour across environments. Similar to the field experiment by Barrett *et al.* (2008), this study used only F1 fish produced from crosses of rare marine *Eda* heterozygotes. When raised in freshwater, stickleback carrying the low allele had increased growth rate relative to those carrying the complete allele. In saltwater this growth advantage was present during juvenile growth but lost during adult growth, suggesting that in this environment stickleback are able to develop full armour plates without sacrificing overall growth rate (Figure 6.5). These experiments show that in freshwater stickleback experience a trade-off between armour and growth, because fish carrying the complete allele have high armour but reduced growth rate. Given the importance of growth for overall fitness (Schultz *et al.*, 1991; Curry *et al.*, 2005; Barrett *et al.*, 2008), this result helps to explain how the low allele could be favoured even in freshwater environments where fish predation is common.

The patterns linking the low *Eda* allele with increased growth rate and survival suggest that positive selection in freshwater can result from both direct and indirect effects of having fewer armour plates. However, some results of these experiments cannot be explained by selection on lateral plates. For instance, although *Eda* heterozygotes are characterized by an intermediate level of armour plating relative to the homozygotes, they did not show intermediate fitness relative to homozygotes in these studies. *Eda* heterozygotes showed lower mortality from insect predation and overwinter conditions than did *Eda* homozygotes (Barrett *et al.*, 2008; Marchinko, 2009) (Figures 7.3B, 6.4C). In contrast, during early life history stages prior to the development of lateral plates, heterozygotes had lower relative fitness than homozygotes (Barrett *et al.*, 2008)

(Figure 6.4C). These anomalous patterns imply that either variation at the *Eda* gene has direct or epistatic effects on other phenotypic traits contributing to fitness, or it is linked to other, unidentified loci affecting fitness. The evolution of plates will therefore be determined not solely by the selective consequences of armour, but also by the other effects of the major gene underlying this trait (e.g., Barrett *et al.*, 2009b), and the effects of genes that are in linkage disequilibrium with it. It would not have been possible to discover the early selection against heterozygotes without knowing the major gene for the phenotypic trait, since selection occurred before it was possible to distinguish between lateral plate morphs. These studies underscore the utility of field experiments to measure selection on genes, which can provide powerful tests of hypotheses for the mechanisms responsible for variation in fitness and also lead to discovery of unanticipated fitness effects.

Conclusions

The availability of new genomic resources has recently made it possible to find the genes contributing to phenotypic variation in natural populations. Incorporating a priori knowledge about the functional properties of genes and their resulting effects on phenotypic traits, organismal performance, and fitness will facilitate a more comprehensive understanding of the role that ecology can play in driving evolutionary patterns (Dalziel *et al.*, 2009). The functional analysis of adaptive variation in three-spined stickleback lateral plate number provides a useful case study showing the power of this approach. This research program has helped to address basic questions about the timing and strength of selection, the genetic architecture of adaptation, the mechanisms driving changes in allele frequency, and the source of genetic variation used in adaptation

to new environments. Significant investment was required to build a suite of new genomic tools for stickleback (Peichel *et al.*, 2001; Colosimo *et al.*, 2004; Cresko *et al.*, 2004; Kingsley *et al.*, 2004; Colosimo *et al.*, 2005; Peichel, 2005; Cresko *et al.*, 2007; Kingsley and Peichel, 2007; Baird *et al.*, 2008), yet these resources have allowed researchers to add a molecular component to a solid foundation of evolutionary and ecological research built over many decades (Bell and Foster, 1994). An exciting prospect for researchers studying other species of fish is that the increasing accessibility of high-throughput sequencing, together with improvements in bioinformatics tools, will make it far less laborious and expensive to develop their own favourite species into a model species.

Future studies investigating the functional properties of candidate genes may be able to identify the specific mutations that cause ecological differences between genetic variants. We have very few cases in wild populations in which a particular molecular change has been functionally associated with an ecologically significant trait (e.g., Hoekstra *et al.*, 2006). Moreover, this level of resolution has only been successful for single major effect genes whereas most trait differences are likely governed by complex interactions between mutations at different genes. Thus, the gap in our knowledge of the molecular interactions responsible for ecologically relevant traits makes it difficult to generate predictive models of adaptive evolution for most variation in nature. The functional analysis of natural variation will benefit greatly from a focus on dissecting "oligogenic" traits (i.e., traits controlled by several, but not hundreds, of genes with major and minor effects) to identify multiple mutations contributing to an adaptive phenotype. Even lateral plate number in stickleback, which is often cited as an example of a "single

locus" trait because of the major effects of *Eda*, is determined by more complicated inheritance patterns than originally thought (Peichel *et al.*, 2001; Colosimo *et al.*, 2004; Cresko *et al.*, 2004; Miller *et al.*, 2007). Increased marker density provided by new genomic tools will greatly facilitate the discovery of minor effect loci and the epistatic interactions between them (Baird *et al.*, 2008). Fine-scale analysis of the genetic architecture underlying lateral plates and other putatively adaptive traits will make it possible to distinguish the effects of physical linkage and pleiotropy in driving the changes in gene frequency that have been observed in selection experiments (Barrett *et al.*, 2008; Cresko, 2008; Barrett *et al.*, 2009a; Marchinko, 2009).

The research program described in this review demonstrates the utility of employing a strategy that starts with a phenotype of interest, identifies genotype, and finally evaluates the fitness consequences arising from the phenotypic effects of specific alleles under natural conditions. This approach can improve the chances that underlying phenotypes and genotypes are relevant for adaptation (e.g., Rogers and Bernatchez, 2007), thus improving our understanding of the ecological mechanisms responsible for evolutionary change in natural populations. With the current molecular revolution, these methods will soon be accessible to researchers studying species from across the broad range of fish diversity.

Figure 6.1. Lateral plate morphs in marine stickleback.

Complete morph (top), partial morph (middle), and low morph (bottom). Fish were stained with Alazarin red to highlight bone.

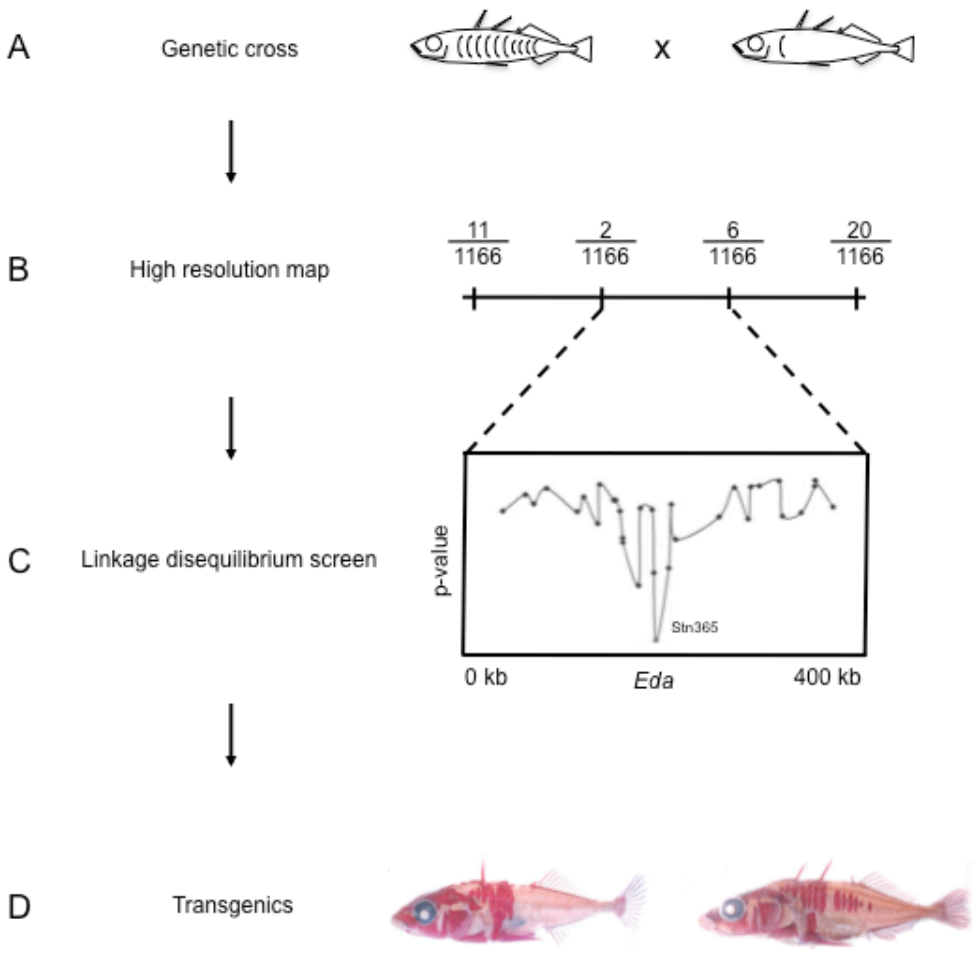

Figure 6.2. Identifying the genetic basis of lateral plate phenotype.

(A) A complete plate morph from the Sea of Japan was crossed with a low plate morph from Paxton Lake in British Columbia. The resulting F1 progeny were then crossed to produce an F2 generation that segregated variation in lateral plate morph. (B) High-resolution genetic mapping identified microsatellite markers that rarely recombine with the plate morph locus (number of recombinants in 1166 chromosomes shown). (C) Linkage disequilibrium screening demonstrated that marker Stn365, located within the *Eda* locus, showed large differences in allele frequency in completely and low-plated fish from Friant, California. (D) Introduction of an *Eda* cDNA construct stimulates lateral plate formation. Fish on the left is a control low plated stickleback and fish on the right is a sibling from the same clutch after introduction of the transgene. Note six extra lateral plates that have formed in the transgenic stickleback (Modified from Colosimo *et al.*, 2005).

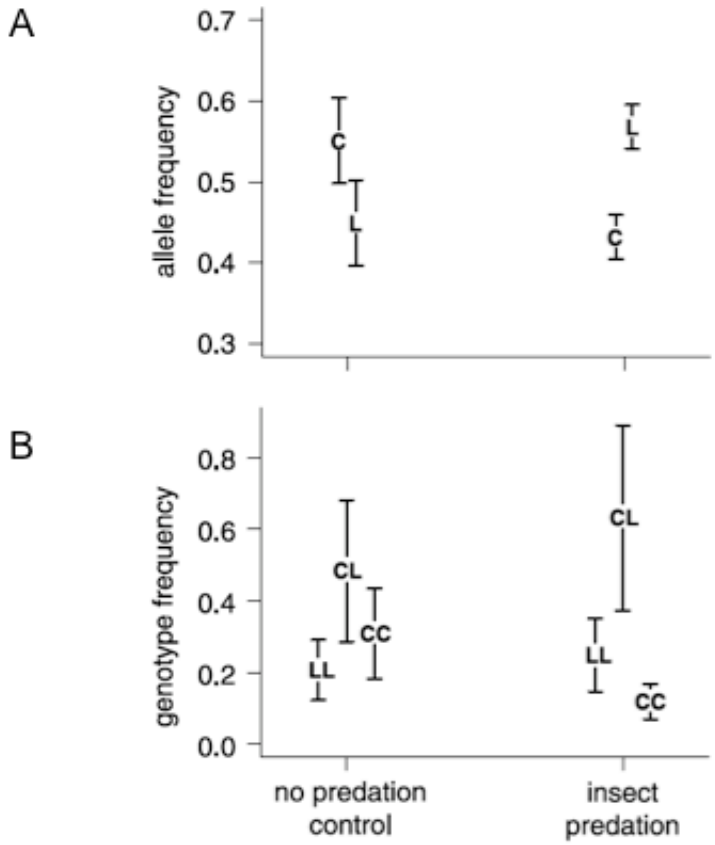

Figure 6.3. Insect predators select against the Eda complete allele.

Allele frequency (A) and genotype frequency (B) at the *Ectodysplasin* locus in F2 juveniles from control and predation treatments. Letters indicate among family mean (± 1 SE). In the top panel, L corresponds to the low allele and C represents the complete allele. In the bottom panel, LL, CL, and CC represent the low allele homozygote, the heterozygote, and complete allele *Eda* genotypes, respectively. Data are from the six F2 families generated from crosses made from fish collected from Paxton Lake (specifically the benthic population) and Oyster Lagoon, British Columbia (Modified from Marchinko, 2009).

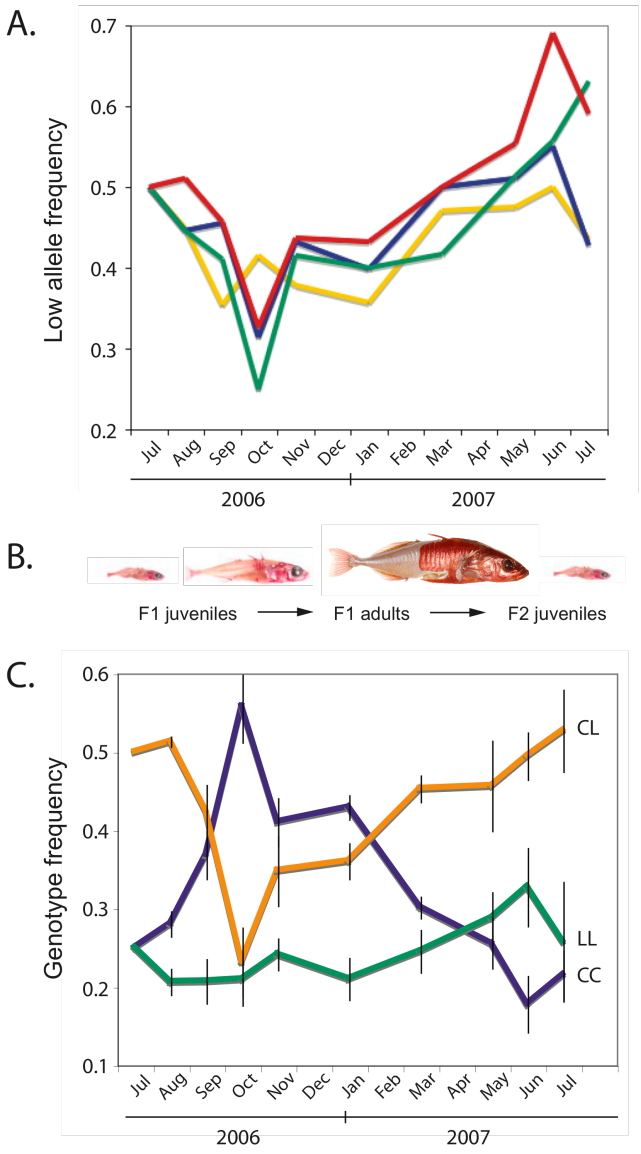

Figure 6.4. Natural selection on Eda *in natural populations of marine sticklebacks transplanted to freshwater ponds.*

(A) Frequency of the low allele in four replicate ponds (different coloured lines). All samples are from the first (F1) cohort of offspring except the June and July 2007 samples, which are from the second (F2) pond generation. (B) Approximate life history stages through the course of the experiment. Fish stained with alazarin red to highlight plate morphology. (C) Genotype frequencies averaged across all four ponds. All samples as in (A). Purple = homozygous complete genotype (CC), orange = heterozygote genotype (CL), and green = homozygous low genotype (LL). Vertical bars show standard errors on the basis of $n = 4$ ponds.

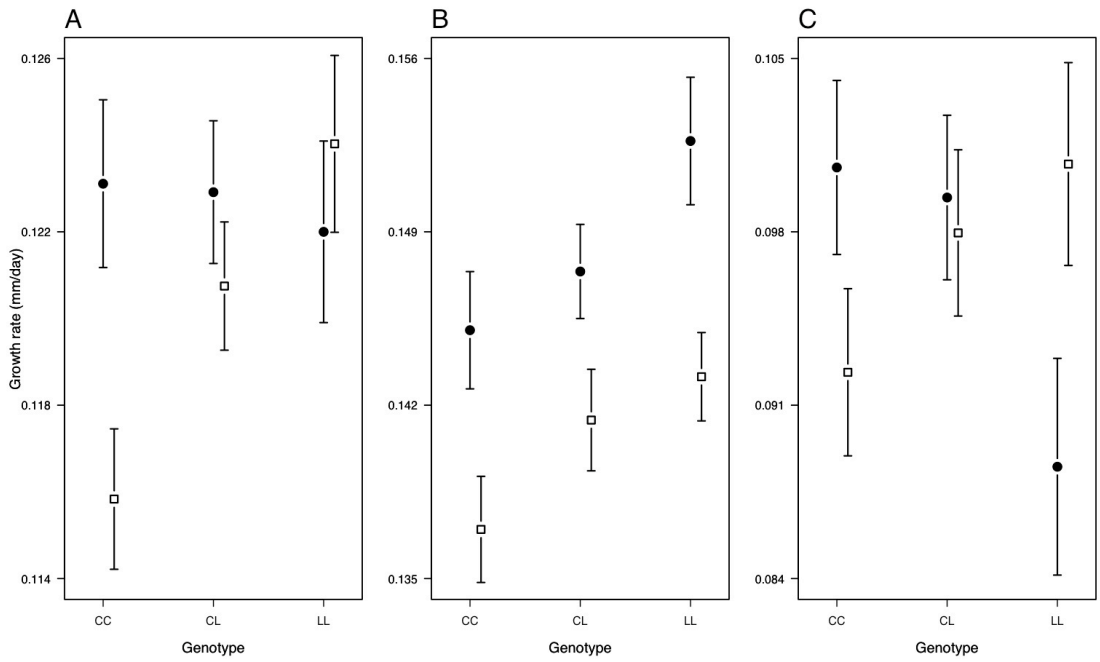

Figure 6.5. Environment specific effects of Eda *on growth rate in salt (●) and fresh water (□).*

(A) Total growth rate by *Eda* genotype (B) Juvenile growth rate by genotype, calculated from birth to the end of adult plate number development at average standard length of 27 mm. (C) Adult growth rate by genotype, calculated between juvenile growth and the end of the experiment at average standard length = 44 mm. Error bars show ± 1 standard error. Note different scale on vertical axis in each panel.

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7. RAPID EVOLUTION OF COLD TOLERANCE IN STICKLEBACK[6]

Introduction

It has been suggested that climate change will have substantial effects on global biodiversity through increased extinction risk for many species (Crowley & North 1988; Deutsch et al. 2008; Hoffmann et al. 2003; Huey et al. 2009; Malcolm et al. 2006; 2009; Thomas et al. 2004). Historically, species have been viewed as relatively fixed entities that cannot evolve in response to changing climate (Skelly et al. 2007). However, accumulating evidence is showing that shifts in climate have led to heritable changes in a wide variety of taxa, rendering the need to incorporate knowledge of evolutionary processes into conservation and management policy (Bell & Collins 2008; Bradshaw & Holzapfel 2006; Smith & Bernatchez 2008). Studies investigating evolutionary responses to climate change have generally involved observation of correlated change between an environmental stress (e.g. temperature) and a phenotypic trait associated with tolerance of the stress (Balanya et al. 2006; Bearhop et al. 2005; Berthold et al. 1992; Bradshaw & Holzapfel 2001; Jonzen et al. 2006; Kausrud et al. 2008; Nussey et al. 2005; Reale et al. 2003; Umina et al. 2005). Experiments are needed to rigorously evaluate the cause and effect relationships underlying adaptation to climate change. Artificial selection experiments have demonstrated that species can be limited in their adaptive potential due to low levels of genetic variation in traits required for survival (Hoffmann et al. 2003; Partridge et al. 1999), but these results may not be applicable to natural populations. Field

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experiments that directly measure rates of evolution in response to natural selection imposed by changes in temperature will help to determine whether wild populations have the ability to adapt rapidly enough to survive climate change. Here, we combine surveys of temperature tolerance in wild populations of threespine stickleback (*Gasterosteus aculeatus*) with laboratory crosses and transplant experiments to show that heritable differences between populations can permit evolutionary responses of sufficient magnitude to permit adaptation to climate change.

Methods

Environmental temperatures

Threespine sticklebacks occur in marine and freshwater environments, which differ in temperature regime. We obtained water temperature data from the British Columbia Lighthouse Data Archive of the Canadian Department of Fisheries and Oceans and the Freshwater Lakes Data Archive of the British Columbia Ministry of the Environment (See Table C.1 for locations). We recorded water temperature from Oyster Lagoon, British Columbia and experimental freshwater ponds at the University of British Columbia, Vancouver using Hobo Data Loggers (The Weather Shop, Westham, UK). All temperatures were recorded from a depth of 2m or less.

Sample populations

We collected adult sticklebacks in April and May 2006, May 2007, and September 2008 from two marine and two freshwater locations in Southwestern British Columbia (Table C.2). Approximately 60 individuals were sampled from each location except Oyster

Lagoon, where we sampled approximately 250 individuals. We transplanted all fish to 102 L glass aquaria. We maintained a density of 15-20 fish per aquaria, salinity of 6-10ppt (gradually decreased to 0ppt within 3 weeks), water temperature of 17±2 °C and a photoperiod of 14h light: 10h dark. To allow individual identification, we injected each fish subepidermally with a fluorescent visible implant elastomer tag (Northwest Marine Technology, Shaw Island, WA, USA) using a 29-gauge syringe.

Crossing design

To test whether any population differences in temperature tolerance are heritable, we generated F1 crosses from within each population (30 families) and also between a marine and a freshwater population (8 families), and reared offspring in the lab under a common constant temperature of 17 °C. To make a cross, we first equally distributed a female's eggs into a Petri dish containing fresh water supplemented with salt (5 ppt, pH 7; Instant Ocean synthetic seasalt, Aquarium Systems, Inc., Mentor, OH, USA). We then sacrificed a male using MS-222 and removed the testes. We placed the testis in a Petri dish and crushed them to release sperm. We left the clutches of eggs and the sperm for 20 minutes and then placed them into separate plastic egg cups (pint cups with fine fiberglass mesh lining the bottom) and submerged each into a separate 102L tank. We added methylene blue to egg tanks to reduce fungal growth and removed any eggs that became inviable due to fungal growth. After eggs hatched and larvae dropped into the tanks, we removed the cups and any unhatched eggs.

Experimental rearing

We fed larvae live brine shrimp twice per day for six weeks and then frozen daphnia and blood worms once per day until 12 weeks of age, followed by a blood worm diet. After feeding stopped we removed any remaining food by filtration or manual siphoning, ensuring that each individual was fed to satiation.

Thermal tolerance testing

All fish were acclimated to lab conditions for a minimum of 3 weeks before they were tested for thermal tolerance. Lab-raised F1s were tested once they reached ~30mm in length. We assessed temperature tolerance using critical thermal maximum (CTMax) and critical thermal minimum (CTMin), defined as the upper and lower temperatures, respectively, at which fish lose the ability to escape conditions that will ultimately lead to death (Beitinger et al. 2000). In the laboratory, CTMax and CTMin are usually estimated as the temperature at which loss of equilibrium occurs following gradual heating or cooling as an empirical endpoint (Fangue et al. 2006). Our experimental set-up consisted of two rectangular plastic water baths (50×35×15cm) each containing 10 individuals in plastic test beakers. The water baths were filled with nitrogen glycol that could be either cooled or heated by adding dry ice or the use of electrical heaters respectively. Cooling and warming rates were maintained between 0.28 and 0.33 °C min^{-1}. We individually aerated each beaker to maintain saturated oxygen concentration and prevent thermal stratification. We continued testing until each fish reached CTMin or CTMax. CTMin values were highly repeatable over varying lengths of acclimation to the lab (Figure C.1).

Repeated CTMax trials could not be run on the same individual because reaching CTMax is sometimes lethal.

Selection experiment

To determine the rate at which cold tolerance can evolve in response to a change in temperature regime, we measured cold tolerance in populations of marine stickleback that had been experimentally introduced to three freshwater ponds two years previously and that had survived two winters in which water temperatures had dropped below the minimum seen in the marine environment (Figure C.2). The ponds are located at the University of British Columbia, Vancouver, British Columbia, Canada and measure 23 m × 23 m with a maximum depth of 3 m in the centre, as described (Schluter 1994). Like many coastal lakes in British Columbia, the ponds are lined with sand and bordered with limestone. All ponds had been previously drained, cleaned and refilled in 2001, allowing plant and invertebrate communities to re-establish, but remaining free of fish until this experiment. The plants and invertebrates used to seed the ponds were collected from Paxton Lake, Texada Island, British Columbia, an 11-ha lake that contains wild sticklebacks. Apart from their construction, initialization, and use in prior experiments, the ponds are unmanipulated environments. In previous experiments these ponds have sustained large populations of sticklebacks over multiple generations, with life cycles and diets characteristic of their wild source populations (Schluter 2003). Growth rates of fish in the ponds are similar to those of wild fish in freshwater lakes (Day et al. 1994).

On June 1, 2006, we introduced marine sticklebacks from Oyster Lagoon into the ponds (pond 1 $n = 45$, pond 2 $n = 46$, pond 3 $n = 46$). This experimental colonization was part of a study aimed at clarifying mechanisms of selection acting on lateral plate armour (Barrett et al. 2008), which is greatly reduced in many freshwater populations relative to marine populations (Colosimo et al. 2005). All fish were heterozygous at the *Eda* locus, a gene that controls lateral plate armour (Colosimo et al. 2005). Within 60 days, we observed larval fish in each colonized pond, indicating that the marine colonizers were breeding. Genotyping of four microsatellite markers confirmed that nearly all alleles present in the parents were at similar frequencies in the progeny, which suggested that founding events did not confer any sampling artefacts (Fisher's combined probability test indicates no significant departure from Hardy-Weinberg equilibrium: parents $\chi^2_{(4)} = 6.303$, $P = 0.178$, progeny $\chi^2_{(4)} = 7.419$, $P = 0.115$). We observed further cohorts of juveniles produced in the ponds in June 2007 and June 2008. In September 2008, we sampled 77 fish (pond 1 $n = 39$, pond 2 $n = 15$, pond 3 $n = 23$) from the third generation (F3) to test for evolved changes in cold tolerance. We compared the cold tolerance of evolved fish to fish sampled from Oyster Lagoon in September 2008. This Oyster Lagoon sample included both heterozygotes and homozygotes at the *Eda* locus. We found no difference between the mean cold tolerance of this Oyster Lagoon sample and the previous sample from May 2007 (contrast = 0.44 [±0.58 95% C.I.]), although there was increased variation in cold tolerance in the 2008 sample (Variance ratio test $F_{(53,27)} = 4.305$, $P < 0.001$). We also found no effect of *Eda* genotype on the cold tolerance of Oyster Lagoon or F3 fish (Oyster Lagoon: ANOVA $F_{(2)} = 0.397$, $P = 0.674$; F3: ANOVA

$F_{(2)} = 0.166$, $P = 0.848$). Before testing, all fish were acclimated in the lab for six weeks at 17 °C.

Statistical analysis

We tested for differences between populations from marine and freshwater by calculating if the mean difference between environment types was greater than the pooled 95% confidence interval. Mean differences were calculated as a vector of constants specifying a linear combination of population means that sum to 1. We used the same method to test for differences between the ancestral population and the F3 generation in the selection experiment.

Results

Wild populations

Lakes are warmer in summer and colder in winter than the sea (Figure C.2). Accordingly, we found significant differences in the cold tolerance between wild marine and freshwater populations (Figure 7.1*a*; mean difference = 2.88 [±0.20 95% C.I.]; see Table C.2 for location of populations). Cold tolerance values for marine and freshwater populations overlapped the minimum environmental temperature experienced in their respective habitats (Figure 7.1*a*). In contrast, heat tolerance values for all populations were considerably higher than maximum environmental temperatures and we detected no significant difference in heat tolerance between marine and freshwater populations (Figure 7.1*b*; mean difference = 0.24 [±0.33 95% C.I.]).

Lab-raised populations

The magnitude of the difference in cold tolerance seen between marine and freshwater populations persisted in the lab-raised F1 generation (Figure 7.2; mean difference = 2.61 [±0.66 95% C.I.]), suggesting that population differences measured after an adequate acclimation period in the lab are not due to phenotypic plasticity caused by environmental temperatures experienced during development. Cold tolerance values were similar between the reciprocal F1 crosses between a marine and a freshwater population, suggesting there were no maternal effects on cold tolerance (Figure 7.2; difference = 0.02 [±1.02 95% C.I.]).

Evolved populations

We observed a strong improvement in cold tolerance of evolved fish relative to the ancestral population, with replicate ponds showing parallel reductions in cold tolerance (Figure 7.3; mean difference = 2.51 [±0.44 95% C.I.]). Across three generations, cold tolerance evolved at an estimated average rate of 0.63 *haldanes* (a *haldane* is equal to a change of one phenotypic standard deviation per generation) to a value 2.5 °C lower than that of the ancestral population, attaining values found in wild freshwater populations (mean difference = 0.52 [±0.84 95% C.I.]). This rate of phenotypic evolution is among the most rapid to be observed in a natural population (Hendry & Kinnison 1999).

Discussion

The ability to tolerate increasingly severe temperature extremes will be crucial for species to adapt to the greater variability in temperature expected from climate change

(Easterling et al. 2000; Gienapp et al. 2008; Lenton et al. 2008; Loarie et al. 2009; Meehl & Tebaldi 2004; UNEP 2009; Schar et al. 2004). Our results suggest that sufficient genetic variation exists in the ancestral marine stickleback population to permit the evolution of a 2.5 °C shift in cold tolerance. However, we caution against interpreting this result as suggesting that natural populations can adapt to climate change without negative consequences. The strong selection required to shift a phenotypic trait so rapidly can result in large changes to population and ecological dynamics that may in turn negatively affect population persistence (Darimont et al. 2009; Kinnison et al. 2008; Yoshida et al. 2003). This cost of rapid adaptation may have been manifested during the winter following our F3 sample, during which all populations went extinct as temperatures reached the lowest minimum recorded in 39 years for the local area (Environment Canada 2010). Alternatively, these extinctions could reflect the limits of adaptation to temperature extremes. The populations may not have been able to evolve cold tolerance low enough to survive the large drop in minimum temperatures, or mechanisms to deal with the indirect effects of cold temperature, such as anoxia caused by ice cover.

This work highlights the utility of transplant experiments for testing the feasibility of rapid evolution in response to climate change. The exceptionally high rates of evolution we observed alter our understanding of the tempo at which temperature tolerance in fish can evolve. It remains to be seen if stickleback populations living in locations with environmental temperatures closer to their maximum heat tolerance will be capable of adapting to shifts toward warmer temperatures. The observed increase in carbon dioxide concentration since 1750 is predicted to cause a minimum warming of

1.4-4.3 °C above pre-industrial surface temperatures (Ramanathan & Feng 2008), suggesting that fish populations that are captive in lakes and unable to migrate northward will require evolutionary responses at least as large as observed in this study to adapt to climate change.

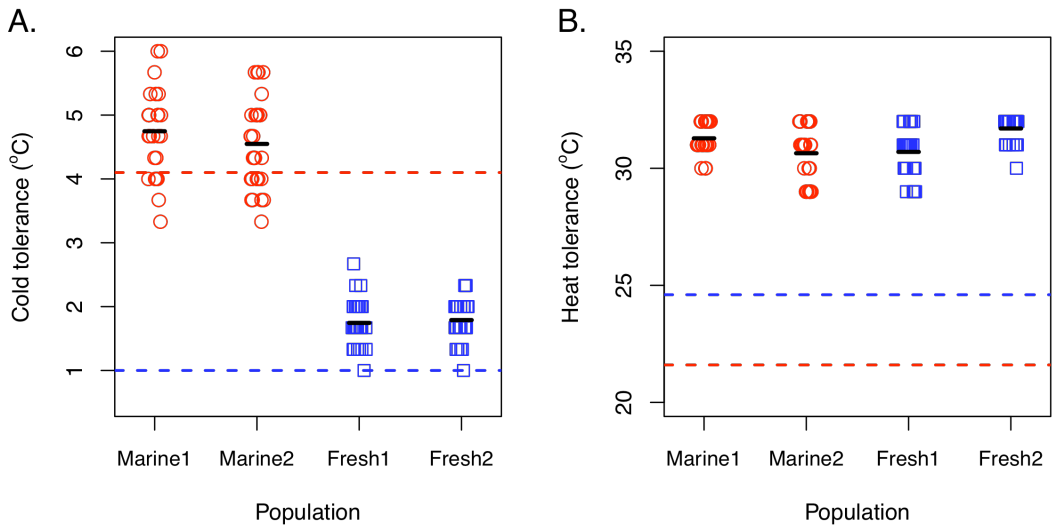

Figure 7.1. Temperature tolerance of wild stickleback.

Red circles and blue squares indicate individual fish from marine and freshwater populations, respectively. (A) Cold tolerance of wild stickleback acclimated to 17 °C in the lab. (B) Heat tolerance of lab-acclimated wild stickleback. Dashed lines show the minimum (A) and maximum (B) temperatures from 11 marine sites (red) and 14 freshwater lakes (blue) in British Columbia (See Table C.2 for locations). Black bars indicate mean values. All fish were tested within 5 days of each other. Marine1 = Little Campbell, Marine2 = Oyster Lagoon, Fresh1 = Cranby Lake, Fresh2 = Hoggan Lake.

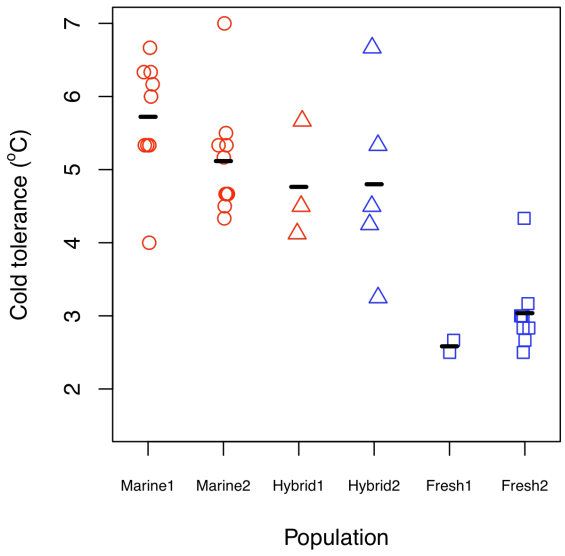

Figure 7.2. Cold tolerance of lab-raised stickleback.

Symbols indicate family averages using 3 or 4 fish from pure marine (red circles), pure freshwater (blue squares) or marine by freshwater (red triangles for marine mother, blue triangles for freshwater mother) crosses. Black bars indicate mean values. All fish were tested within 5 days of each other. Marine1 = Little Campbell, Marine2 = Oyster Lagoon, Hybrid1 = Oyster Lagoon by Cranby Lake, Hybrid2 = Cranby Lake by Oyster Lagoon, Fresh1 = Cranby Lake, Fresh2 = Hoggan Lake.

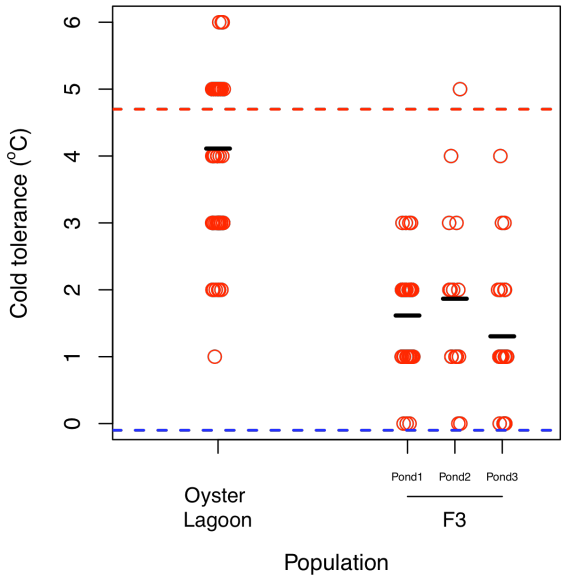

Figure 7.3. Rapid evolution of cold tolerance in a marine population of stickleback transplanted to freshwater.

Circles indicate individuals from the ancestral population (Oyster Lagoon) for the selection experiment and the F3 generation in the ponds. Dashed lines show the minimum temperature from Oyster Lagoon, British Columbia (red) and averaged from 3 ponds located at the University of British Columbia, Vancouver, British Columbia (blue). All fish were sampled in September 2008, acclimated for six weeks in the lab at 17°C and tested within 5 days of each other. Black bars indicate mean values.

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8. THE DISTRIBUTION OF BENEFICIAL MUTANT EFFECTS UNDER STRONG SELECTION[7]

Introduction

Populations adapt to their environments through the appearance and subsequent spread of random beneficial mutations. In a sexual population, recombination can bring together beneficial mutations that arise in different lineages. In asexual populations, however, mutations can only fix sequentially (Novick and Szilard 1950; Atwood *et al.* 1951; Crow and Kimura 1965). Distinct genotypes cannot recombine and instead must compete with each other, a phenomenon known as "clonal interference" (Gerrish and Lenski 1998; Miralles *et al.* 1999; de Visser and Rozen 2006). Thus, for a mutation to contribute to adaptation it must not only escape sampling error (drift), but it must also fix before being eliminated by the occurrence and more rapid sweep of a superior mutation.

Theory for determining the probability that selection will fix a new favourable mutation was first formulated 80 years ago by Fisher (1922) and Haldane (1927; 1930), who focused on the fate of a single isolated mutation. In a recent flurry of papers, researchers have explored the distribution of fitness effects expected among the array of possible beneficial mutations that might arise within a population (Gillespie 1983; Gillespie 1984; Gillespie 1991; Orr 2002; Orr 2003). This distribution can be seen as the starting point for progress toward a general theory of adaptive evolution. From this first distribution, we can determine the distribution of those mutations not lost by drift, referred to as "contending mutations" (Gerrish and Lenski 1998; Rozen *et al.* 2002). The

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distribution of contending mutations can subsequently be used to determine the distribution of mutations that outcompete other genotypes and fix in a population ("fixed mutations"), thus contributing to adaptation (Orr 1998; Rozen *et al.* 2002).

In addition to theory, recent empirical studies have examined the steps from newly arisen mutations, to contending mutations, to fixed mutations. The underlying distribution of fitness effects of beneficial inferred from experiments is generally consistent with an exponential distribution (Imhof and Schlotterer 2001; Rozen *et al.* 2002; Sanjuan *et al.* 2004; Kassen and Bataillon 2006), while the final distribution of fixed beneficial mutants appears roughly bell-shaped (Rozen *et al.* 2002; Rokyta *et al.* 2005; Barrett *et al.* 2006). These empirical results must be interpreted cautiously, however, as there is typically little power to reject other distributions (e.g., more L-shaped or more bell-shaped distributions, see (Kassen and Bataillon 2006).

An important caveat to the theoretical side of this work, however, is that it has assumed weak selection. R.A. Fisher (1930) first justified this assumption using the analogy of movement from the outer surface of a sphere (representing phenotype space) to an optimum at the center; Fisher argued that mutations of small size have a 50% chance of bringing the population closer to the optimum, while larger mutations have a rapidly diminishing probability of being advantageous. This argument led to a "gradualist" view of adaptation, in which evolutionary change overwhelmingly proceeds through the selection of very slightly beneficial alleles (Orr 2005). Assumptions of weak selection common to all diffusion equations and many other theoretical approaches make it difficult to predict the dynamics of strongly beneficial mutations (Morjan and Rieseberg 2004). Simulations have demonstrated that although fixation times for strongly

advantageous alleles are accurately predicted by diffusion, the probability of fixation is underestimated as the strength of selection increases (Whitlock 2003).

In recent empirical studies, researchers have observed mutations with very high selection coefficients (Bull *et al.* 2000; Barrett *et al.* 2006), especially when organisms face novel environments. Strong selection has an important impact on the theory of adaptation. In particular, the fixation probability of mutations and the number of competing mutations will be highly dependent on the fitness effect of the mutation in question (Rozen *et al.* 2002). When selection is assumed to be weak, new mutations remain at low frequency for a considerable period of time before reaching fixation (Gerrish and Lenski 1998). This provides ample opportunity for beneficial mutations to be lost and for competing mutations to arise (Gerrish 2001). In contrast, when selection is strong, the probability of fixation approaches its maximum value of one and the time to fixation is relatively short, reducing the number of competing mutations and the importance of clonal interference. Yet previous predictions cannot be applied to the case of strong selection, because of the pervasive theoretical assumption that selection is weak.

Here, we derive population genetic theory to describe the impact of drift and clonal interference on the fitness distribution of fixed beneficial alleles without the assumption of weak selection. We derive these distributions for a wide range of selection coefficients and test our analytical theory against numerical simulations.

The Model

In the following, we describe the probability density functions (pdf) of the selection coefficient, s, among newly arising beneficial mutations, contending beneficial mutations, and fixed beneficial mutations. Following Rozen et al. (2002), we denote these pdfs as $f(s)$, $g(s)$, and $h(s)$, respectively. Where needed, we use capital letters to refer to the corresponding cumulative density functions (cdf) ($F(s)$, $G(s)$, and $H(s)$, respectively). Throughout, we assume that the population size is large, haploid, and asexual. The results may be applied to asexual diploids by replacing s with $h\,s$, where h is the dominance coefficient and mutations are assumed to fix in the heterozygous condition. An extension to sexual diploids is straightforward (at least numerically), but it requires that the joint distribution of h and s be specified.

The distribution of beneficial mutations

It is generally assumed that the wild type has very high fitness and almost all mutations are deleterious (Gillespie 1983; Gillespie 1984; Orr 1998). It follows that beneficial mutations will lie in the extreme right tail of a distribution of all mutant fitness effects. This inference justifies the application of extreme value theory (Gumbel 1958) to describe the distribution of beneficial mutant effects. Extreme value theory suggests that the distribution of mutant effects, restricted to beneficial mutations, will be nearly exponential (Gillespie 1983; Gillespie 1984). This requires, however, that only a tiny minority of mutations are beneficial. In very harsh or novel environments, mutations that were previously deleterious may become beneficial, thus increasing the size of the beneficial mutant class. In these situations, extreme value theory may not hold and an

exponential distribution might not be an adequate description of the selective effects of new mutants.

While empirical and theoretical studies indicate that the exponential distribution is a plausible distribution describing the fitness effects of new beneficial mutations, distributions with other shapes cannot be rejected and might be more appropriate under certain circumstances. Thus, to allow greater flexibility, we assume that the selection coefficients of new beneficial mutants, *s*, follow a gamma distribution with mean selection coefficient, σ, and coefficient of variation, *cv*:

$$f(s) = \frac{e^{-\frac{s}{cv^2\sigma}} \left(cv^2\sigma\right)^{-1/cv^2} s^{-1+1/cv^2}}{\Gamma\left[1/cv^2\right]} \quad (1)$$

where $\Gamma[a]$ is Euler's gamma function. The shape of the gamma distribution varies from L-shaped (high *cv*) to bell-shaped (low *cv*), allowing a broader range of distributions to be described. The exponential distribution represents a specific case of the gamma where the coefficient of variation equals one.

The distribution of contending beneficial mutations

We begin by deriving the distribution of fitness effects among those contending mutations that survive stochastic loss while rare. Haldane (1927) used a branching process to show that *P*, the probability of fixation, satisfies $1 - P = e^{-(1+s)P}$ in populations of constant size when the number of offspring per parent is Poisson distributed. Using a diffusion approximation, Kimura (1957; 1964; 1983) extended this theory for populations of finite size, *N*, showing that $P \approx \frac{1 - e^{-2s}}{1 - e^{-2Ns}}$. For weak selection ($1/N << s << 1$), both of

these equations yield the same approximate fixation probability: $P \approx 2s$. When strong selection is possible, however, a more accurate approximation for both equations is: $P = 1 - e^{-2s}$, obtained by letting N get large in the diffusion result (Figure 8.1). These results assume that a mutant allele is either lost or fixed before other mutations arise. When the mutation rate is sufficiently high, however, a mutation may survive loss while rare, but eventually be outcompeted by mutations arising in the future. In this case, P describes the probability of surviving stochastic loss while rare, not the ultimate fixation probability, which depends on the nature of future mutations.

Because newly arisen mutations have a probability of surviving stochastic loss while rare of approximately $1 - e^{-2s}$ in populations of large size, the distribution of selection coefficients among contending mutations becomes:

$$g(s) = \frac{f(s)(1 - e^{-2s})}{\int f(s)(1 - e^{-2s})\, ds} \tag{2}$$

The denominator represents the probability of surviving drift averaged across the distribution of new mutational effects, Π:

$$\begin{aligned} \Pi &= \int f(s)\,(1 - e^{-2s})\, ds \\ &= 1 - \left(1 + 2\, cv^2\, \sigma\right)^{-1/cv^2} \end{aligned} \tag{3}$$

Using this result the pdf of the selection coefficients among contending mutations is:

$$g(s) = \frac{e^{-\frac{s}{cv^2\sigma}} \left(cv^2\sigma\right)^{-1/cv^2} s^{-1+1/cv^2} \left(1 - e^{-2s}\right)}{\Gamma\left[1/cv^2\right]\left(1 - \left[1 + 2cv^2\sigma\right]^{-1/cv^2}\right)} \tag{4}$$

Because the probability of surviving loss while rare, $P = 1 - e^{-2s}$, asymptotes at one (Figure 8.1), the distribution $g(s)$ is similar to the prior distribution $f(s)$ for mutations of large effect.

The distribution of fixed beneficial mutations

Rozen et al. (2002) defined the expected number of contending mutations arising within other genetic backgrounds before the fixation of a focal mutation as

$$\lambda(s) = PN\mu\frac{T}{2} \tag{5}$$

where P is the average probability of surviving loss while rare, N is the population size, μ is the beneficial mutation rate, and T is the average amount of time until fixation (the 1/2 reflects the fact that, by symmetry, half of the population will not carry the focal mutation when averaged over the period of time during which the focal mutation rises from a single copy to fixation; see Figure D.1). Rozen et al. (2002) then calculated $\lambda(s)$ under the assumption of weak selection. To relax this assumption, we use the general solution for the deterministic haploid model, $q(t)/p(t) = (1+s)^t\, q(0)/p(0)$, where $p(t)$ and $q(t)$ are the frequencies of non-mutant and mutant individuals, to solve for the time taken for an allele initially at frequency $1/N$ to reach a frequency of $1-1/N$. This gives $T = 2\ln(N-1)/\ln(1+s) \approx 2\ln(N)/\ln(1+s)$, which can be used along with $P = 1 - e^{-2s}$ in (5) to estimate the number of contending mutations:

$$\lambda(s) \approx \left(1 - e^{-2s}\right) N\,\mu\,\frac{\ln(N)}{\ln(1+s)} \tag{6}$$

When there are n contending mutations and one focal mutation, each with a selection coefficient drawn from a cumulative density function (cdf) given by $G(s)$, the

cumulative density function of the highest of the selection coefficients is $G(s)^{n+1}$ (Rice 1988). Assuming that the number of contending mutations that appear during the spread of a focal mutation follows a Poisson distribution, d_n, with mean $\lambda(s)$, the cdf for the selection coefficient of the most advantageous of the contending and focal mutations is:

$$H(s) = N^{-N\,\mu\,\kappa/\ln(1+s)}\left(1-\frac{\kappa}{\Pi}\right) \tag{7}$$

where Π is the average probability of fixation (equation 3) and

$$\kappa = \frac{\Gamma\left[\frac{1}{cv^2}, \frac{s}{\sigma\, cv^2}\right]}{\Gamma\left[\frac{1}{cv^2}\right]} - (1-\Pi)\frac{\Gamma\left[\frac{1}{cv^2}, \frac{s\,(1-\Pi)^{-cv^2}}{\sigma\, cv^2}\right]}{\Gamma\left[\frac{1}{cv^2}\right]}. \tag{8}$$

If newly arising beneficial mutations follow an exponential distribution ($cv = 1$), these coefficients simplify to:

$$\Pi = \frac{2\,\sigma}{1+2\sigma} \text{ and } \kappa = e^{-s/\sigma} - \frac{e^{-2s-s/\sigma}}{1+2\sigma}.$$

This equation only accounts for contending mutations that arise after the focal mutation, as no improvement in fit was observed when accounting for prior mutations (data not shown). (Essentially, we consider the first contending mutation to be the focal mutation.) The corresponding probability density function for fixed mutations is then $h(s) = \mathrm{d}H(s)/\mathrm{d}s$.

Numerical simulations

We compare the above analysis to explicit numerical simulations using a Wright-Fisher model (Ewens 1979). We tracked all beneficial mutants segregating in an asexual haploid population of constant size N until a fixation event. Each generation, the number of new

mutations appearing within the population was drawn at random from a Poisson distribution with mean $N\mu$. Each mutation was then randomly assigned a selection coefficient drawn from a gamma distribution with mean σ and coefficient of variation *cv* and assigned a unique identifier. Multiple mutations had independent effects on fitness (no epistasis on a multiplicative scale). Offspring were then sampled with replacement according to a multinomial distribution from the parental distribution of genotypes, weighted by the fitness of these genotypes. A fixation event was defined as the first point in time when all individuals in the population shared a common mutation (with the same identifier). Similar results were obtained when we recorded data for the fifth mutation rather than the first mutation to fix (data not shown). At this point, the process was stopped and the selection coefficient of the fixed mutant was recorded. This selection coefficient was defined as the fitness effect of the fixed mutant when placed in the ancestral background; the average fitness within the population at the time of fixation was also recorded.

To evaluate the robustness of the analytical results, we ran simulations with every combination of the following average selection coefficients (σ : 0.01, 0.1, 1, 2, 10), beneficial mutation rates ($\mu = 10^{-5}, 10^{-7}, 10^{-9}$), population sizes ($N = 10^5, 10^6, 10^7$), and coefficients of variation ($cv = 0.5, 1, 2$). Simulations were carried out in *Mathematica* (Wolfram Research 2005; available upon request).

Numerical results

The numerical simulations closely match the predictions from our model across the parameter range explored (Figure 8.2). In contrast, analyses based on weak selection

(Rozen *et al.* 2002) consistently overestimate the selection coefficient among fixed mutations (Figure 8.2). Assuming a fixation probability of 2*s* gives unrealistically high fixation probabilities for mutants with large selection coefficients, which inflates the proportion of contending and fixed mutations of large effect.

Analytical and numerical results both predict bell-shaped distributions for the selection coefficients among fixed beneficial mutations (Figure 8.3). The distribution of contending mutations, $g(s)$ (thin solid curves), is always bell-shaped because weakly selected mutations are likely to be lost while rare (Kimura 1983). With a low mutation rate (top panels), the distribution of fixed mutations (histogram) is very nearly equal to the distribution of contending mutations, $g(s)$, and clonal interference has little effect. With a higher mutation rate (bottom panels), clonal interference becomes more important, and only the most fit of the contending mutations fixes within the population, shifting the distribution of fixed mutations to the right. When selection is, on average, stronger (right panels), mutations are less likely to be lost through stochastic drift while rare, causing the distribution of fixed beneficial mutations to be more similar to the distribution of selection coefficients among newly arising mutations, $f(s)$ (dotted curves); consequently, the mean and coefficient of variation among mutations that fixed in the simulations are more similar to the original mean, σ, and *cv* (inset boxes). The shape of the contending and fixed distributions is also influenced by the shape of the distribution of underlying beneficial mutations (Figure D.2). Increasing the coefficient of variation of beneficial mutations results in more contending mutations of large and small effect, increasing the variation observed among fixed mutations.

If there is a high input of new mutations, it becomes more likely for several beneficial mutations to coexist in a population. This leads to clonal interference, as beneficial mutations in different genetic backgrounds compete with one another. In addition, however, a high mutation rate also makes it more likely that multiple beneficial mutations will arise in the same background as previous beneficial mutations. In this case, several beneficial mutations can assist each other's spread to fixation, and the combined fitness advantage from these mutations will be higher than the fitness advantage conferred by the single original mutation. To assess the importance of assisted fixation, we measured the average fitness advantage in the population at the time a beneficial mutation fixed (relative to the non-mutant ancestor). The average fitness advantage was greater than the fitness advantage conferred by the mutation alone (Figure 8.4), often by orders of magnitude when the mutation rate was high enough to expect clonal interference ($N\mu > 1$). As expected, this discrepancy was caused by the effects of additional beneficial mutations segregating within the population at the time of fixation.

We have so far assumed a constant population size, but many experiments designed to detect beneficial mutations involve repeated bottlenecks and a fluctuating population size. Such fluctuations dramatically increase the chance of loss of beneficial mutations, so that only the most favourable alleles are likely to fix. In the Appendix we modify the theory developed above in order to describe how fluctuating population size alters the fixation probability and the time to fixation. We then estimate the number of competing mutations, $\lambda(s)$, and the distribution of fixed mutations, $h(s)$.

Discussion

Based on his geometric model of the adaptive process, Fisher (1930) argued that mutations of very small effect have a nearly 50% chance of pointing toward an optimum, while mutations of very large effect rarely will. This reasoning underlies the common assumption in population genetics that adaptation consists of fine tuning the phenotype with mutations of relatively small effect. What constitutes a large mutation in Fisher's model depends, however, on the fitness of the original population. If a population is initially poorly adapted (e.g., following a recent change in the environment), even major mutations with a substantial effect on phenotype have a nearly 50% chance of pointing toward the optimum. Thus, strongly selected mutations may very well contribute to the process of adaptation, especially during the early stages of adaptation to a novel environment. Furthermore, by virtue of their size, large effect mutations will have a disproportionate influence on the process of adaptation. Data from genetic analyses of quantitative trait differences (Bradshaw *et al.* 1998; Wang *et al.* 1999; Colosimo *et al.* 2005) and from experimental evolution studies (Bull *et al.* 2000; Barrett *et al.* 2006) confirm that mutations with large phenotypic and fitness effects can occur and contribute to the process of adaptation. In this article, we have generalized existing theory about the distribution of fitness effects among fixed beneficial mutations so that it can be applied to situations with strong selection.

The distribution of fitness effects among fixed beneficial mutations is generally derived from the distribution of fitness effects among all possible beneficial mutations, about which little is known. Several theoretical studies have suggested that new beneficial mutations should be exponentially distributed (Mukai *et al.* 1972; Orr 1998;

Rozen *et al.* 2002; Wilke 2004), based on the fact that beneficial mutations represent the tail of the distribution of potential mutant effects (Gillespie 1983; Gillespie 1984). In a novel environment, however, more mutations are likely to be beneficial and the applicability of such extreme value theory is uncertain. We have thus employed a gamma distribution to describe the fitness effects of possible beneficial mutations. Because the gamma distribution has two parameters (described by the mean selection coefficient, σ, and the coefficient of variation, *cv*), we can explore a broader range of possible distributions of mutational effects. We find that the shape parameter of the gamma distribution among newly arising mutations influences the distribution of mutations that survive stochastic loss while rare ("contending mutations") and the distribution of mutations that survive clonal interference to become fixed ("fixed mutations"), especially when selection is strong (Figure D.2). This result appears to contradict a recent study, which reported that the distribution of mutational sizes for fixed mutations is virtually independent of the underlying distribution of beneficial mutations (Orr 1998; Hegreness *et al.* 2006). The simulations run by Hegreness et al. (2006) cover only a range of parameters, within which clonal interference is severe (the population size was set to 2×10^6 and the mutation rate was 10^{-5}). Indeed, using their combination of parameter values in equation (7) indicates that the shape of the distribution of fixed beneficial mutations is nearly independent of the shape of the distribution of newly arising beneficial mutations. Furthermore, using their parameters, most fixed mutations have similar selection coefficients, as pointed out by Hegreness et al. (2006), unlike the fairly broad distributions observed in Figure 8.3.

Beneficial mutations that survive stochastic loss while rare tend, on average, to have a larger fitness benefit, and their distribution tends to have a lower coefficient of variation (more bell-shaped), because very weakly selected alleles are unlikely to fix (Kimura 1983; Gerrish and Lenski 1998; Orr 2000; Otto and Jones 2000; Rozen *et al.* 2002; Wilke 2004). While this is generally true, the effect is less pronounced when selection is strong. That is, the distribution of fixed beneficial mutations is more similar to the distribution of newly arising mutations (Figure 8.3). Consequently, for empirical data involving high selection coefficients, using theory that assumes weak selection will tend to underestimate the mean selection coefficient among newly arising beneficial mutations.

One of the major impediments to theoretical studies of the distribution of fitness effects of fixed beneficial mutations has been a lack of knowledge of realistic parameter values. This is largely because the low frequency of fixed beneficial mutations has prevented empirical work with statistical power. In recent years, however, the use of microbial microcosms has provided a way to increase the number of beneficial mutations likely to arise and fix during an experiment. Three experiments have characterized the distribution of fixed beneficial mutant effects (Rozen *et al.* 2002; Rokyta *et al.* 2005; Barrett *et al.* 2006). All used roughly the same experimental protocol: a number of replicate bacterial or viral lines were introduced into a novel environment and evolution proceeded through the substitution of novel beneficial mutations. By comparing the fitness of an evolved genotype sampled from around the time a mutation fixed within the evolved population to the fitness of the ancestral genotype, these studies claimed to measure the fitness advantage conferred by the single beneficial mutation carried by each

evolved genotype. Our simulations indicate, however, that the selection coefficient estimated from the average fitness of individuals at the time of fixation is a very poor measure of the fitness effect of the actual mutation that has just fixed whenever $N\mu > 1$ (Figure 8.4). Whenever multiple mutations arise during the spread of a focal mutation, so that clonal interference occurs, individuals are likely to carry multiple mutations by the time that the focal mutation has fixed, and therefore selection coefficients measured will overestimate the true effects of a single mutation. Consequently, experiments aimed at estimating the distribution of beneficial selective effects should avoid large population size in order to keep $N\mu < 1$.

The studies by Rozen et al. (2002) and Barrett et al. (2006) serve as a good comparison of how the distribution of fitness effects of fixed beneficial mutations shifts with increasing selection. Both experiments were conducted with similar organisms and transfer protocols and therefore share fairly comparable parameter values, except for the average selection coefficient, which differed by an order of magnitude. This difference is perhaps unsurprising as the ancestral strain in the Rozen et al. (2002) experiment was fairly well adapted to consuming the sole carbon source (*Escherichia coli* with glucose), whereas the ancestral strain in the Barrett et al. (2006) experiment initially had very poor growth (*Pseudomonas fluorescens* with serine). Despite the difference in average selection coefficients, both studies reported bell-shaped distributions for fixed beneficial mutations. Both sets of authors suggest that this shape is the result of drift and clonal interference transforming an exponential distribution of beneficial mutations. However, since $N_e\mu < 1$, the bell-shaped distributions are unlikely to be strongly influenced by clonal interference. Indeed, the bell-shaped distributions observed in these studies can be

accounted for entirely by the stochastic loss while rare of mutations drawn from an exponential distribution (equation 4), without considering competing mutations.

In conclusion, allowing for strong selection has altered our theoretical understanding of the distribution of fitness effects in the following ways. By correctly accounting for the fact that the fixation probability cannot rise above one, the distribution of fixed beneficial mutations more closely matches the distribution of newly arising beneficial mutations when selection is strong (Figure 8.3). Although the distribution of surviving mutations is always more bell-shaped, the difference from the distribution of newly arising beneficial mutations is largely confined to regions where selection is weak. Importantly, our results (Figure 8.4) also demonstrate that data on the selection coefficients of fixed mutations must be treated with caution whenever clonal interference is present, as multiple mutations are likely to be segregating at the time of fixation, causing selection coefficients to be greatly exaggerated.

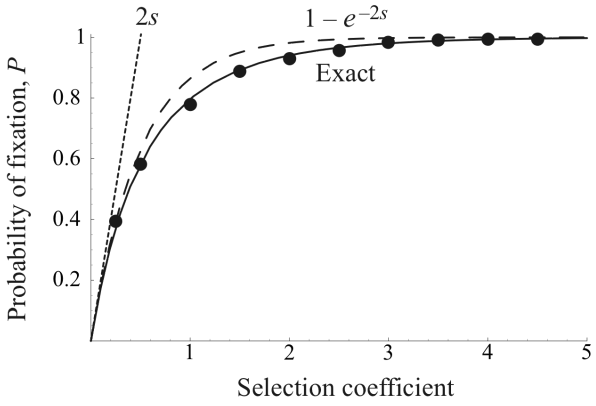

Figure 8.1. The probability of fixation for a beneficial mutation as a function of its selection coefficient.

The dots show the probability of fixation of a single mutant with a given selection coefficient in Wright-Fisher simulations of a large population ($N = 10^6$; based on 1000 replicates). The solid curve shows the exact fixation probability derived by Haldane (1927) using a branching process. The short-dashed line shows the common weak selection approximation $P \approx 2s$. The long-dashed curve shows the approximation used in this paper, $P = 1 - e^{-2s}$, which is indistinguishable from Kimura's diffusion result for these parameters.

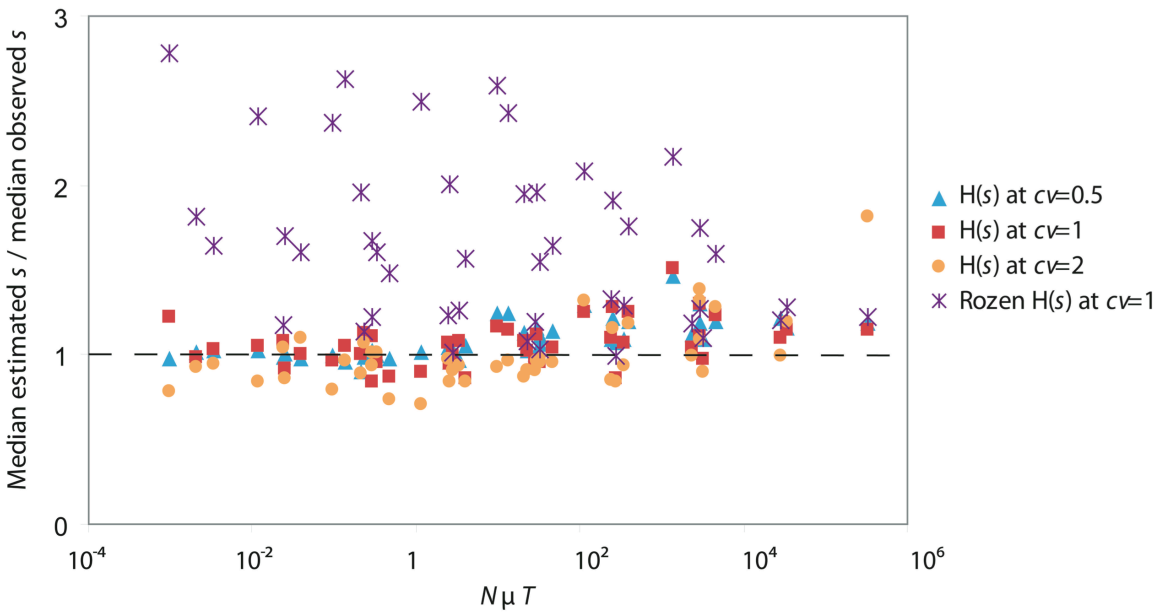

Figure 8.2. Median selection coefficient of fixed beneficial mutations estimated from analytical results versus numerical simulations.

Median estimated s is given from the results of either our generalized selection model, given by $H(s)$ in equation 7, or from a model assuming weak selection and a cv of 1 (equation 2 in Rozen et al. (2002). The horizontal axis measures the degree of clonal interference and equals the number of mutations that appear within the population over the time to fixation, T, for a new mutation with selection coefficient given by the median observed s. Based on 200 replicate simulations per parameter combination.

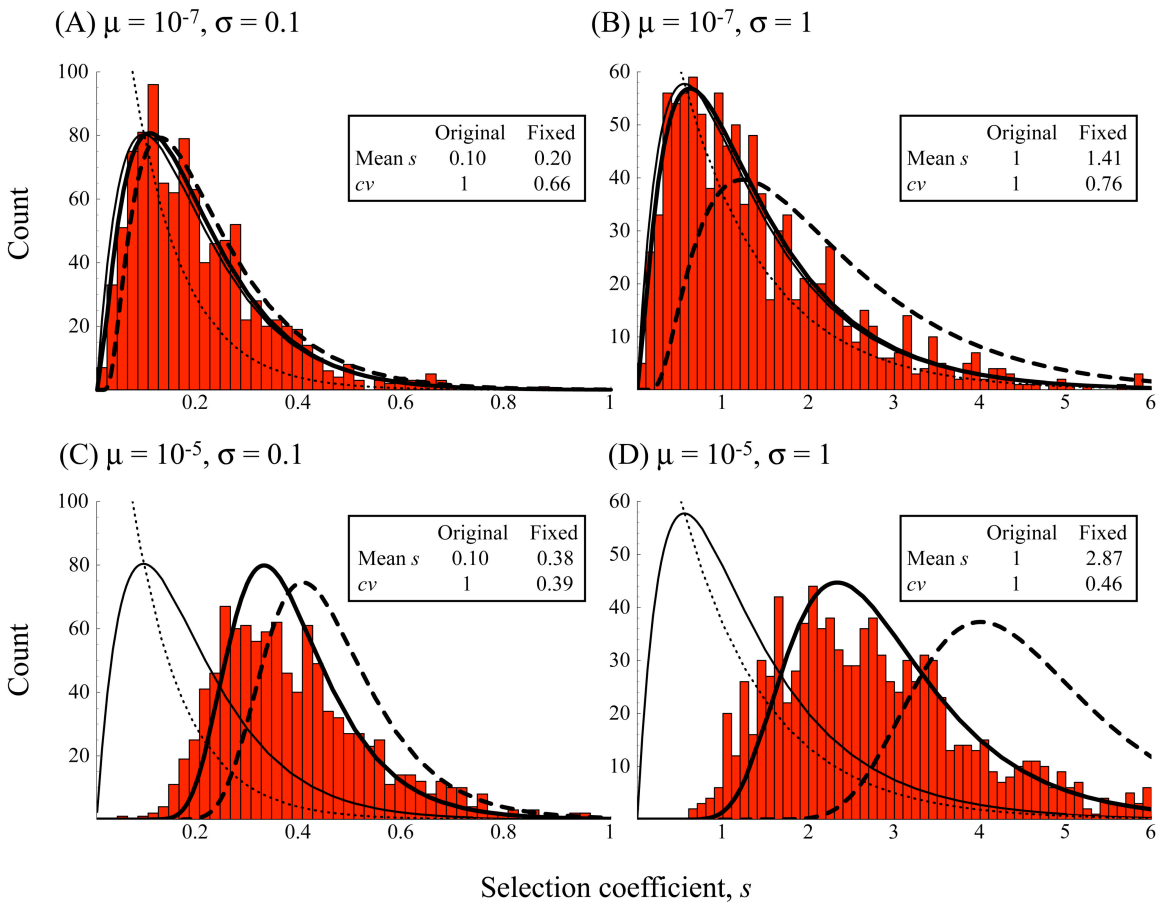

Figure 8.3. Distribution of fitness effects.

The histogram was generated from the fitness effects of fixed beneficial mutations among 1000 replicate simulations; fixed mutations have a higher average selection coefficient and exhibit less variation than newly arising mutations (inset boxes). New mutations were distributed according to an exponential distribution, $f(s)$ (thin dotted curve). Contending mutations that survived loss while rare (thin solid curve) are shifted to the right ($g(s)$ from equation 4). Fixed mutations (thick solid curve) that survived clonal interference are even further shifted to the right ($h(s)$ from the derivative of equation 7) but not as much as predicted using the weak selection approximation given by equation 2 of Rozen et al. (2002) (thick dashed curve). Clonal interference is more important in the bottom two panels, where mutation rates are higher (top: $\mu = 10^{-7}$, bottom: $\mu = 10^{-5}$). Selection is weaker, on average, in the left panels ($\sigma = 0.1$) than the right ($\sigma = 1$). Remaining parameters: $cv = 1$ and $N = 10^5$.

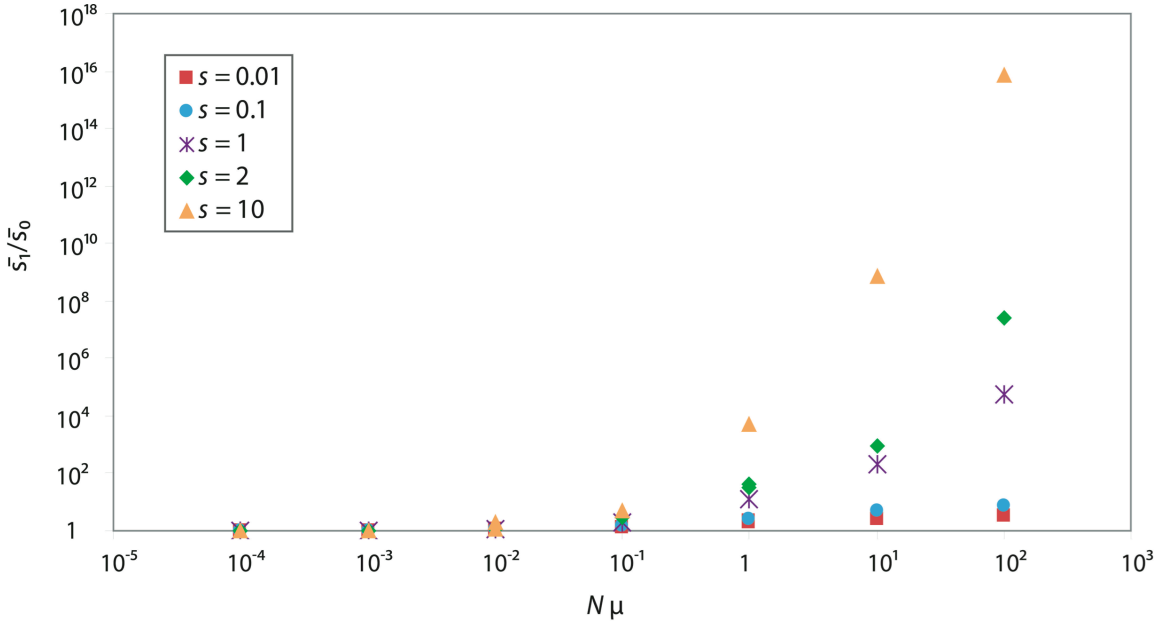

Figure 8.4. Error caused by measuring the selection coefficient using the average fitness at the time of fixation.

The vertical axis gives the amount by which the average fitness in the population has improved over the ancestor when the focal mutation fixes, $\bar{s}_1$, relative to the selection coefficient of that mutation alone, $\bar{s}_0$. The horizontal axis gives the input rate of new mutations, $N\mu$. Based on 200 replicate simulations.

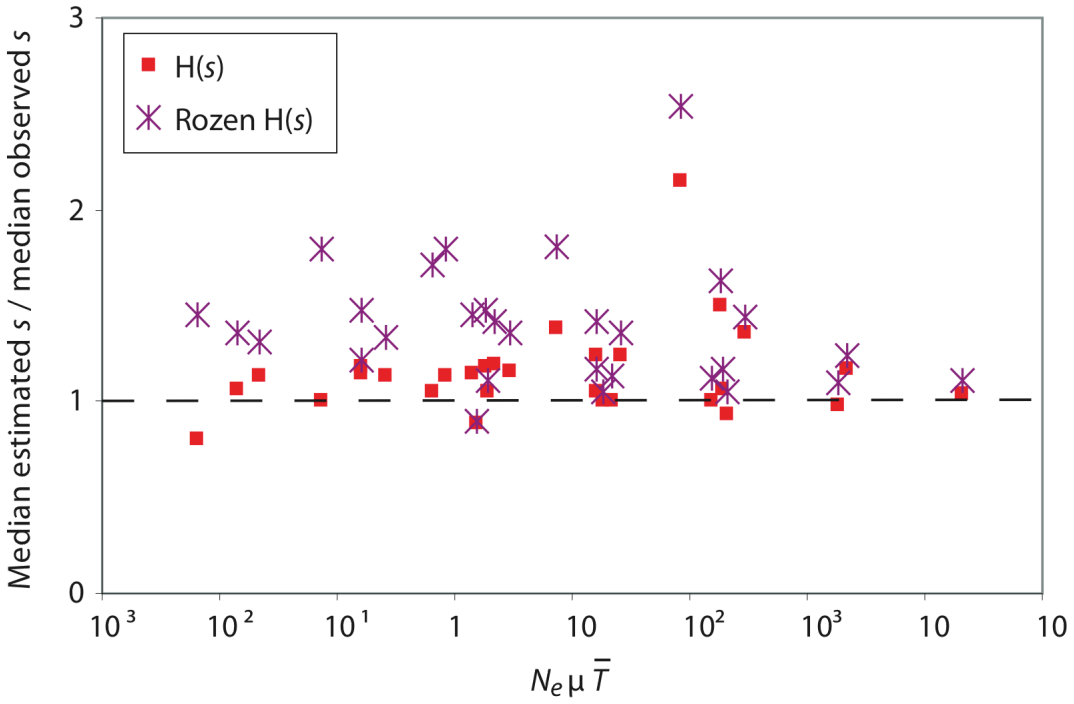

Figure 8.5. Median selection coefficient of fixed beneficial mutations estimated from numerical simulations versus analytical results.

Median estimated s is given from the results of our fluctuating population size model, given by $H(s)$ in equation 11, or from a model assuming weak selection given from equation 2 in Rozen et al. (2002), but replacing N with N_e from Wahl et al. (2002). The horizontal axis measures the number of mutations that appear within the population over the average time to fixation, $\overline{T}$, for a new mutation with selection coefficient given by the median observed s.

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9. CONCLUSIONS

Future directions in studying the genetics of adaptation

After a delay of a few decades while focus was diverted to testing the neutral theory (Kimura 1983), the study of adaptation is once again receiving the level of attention that occurred in the century following publication of Darwin's *On the Origin of Species*. In particular, the development of new theoretical models (reviewed in Orr 2005a, b; Waxman and Gavrilets 2005; Orr 2009) and empirical approaches (reviewed in Feder and Mitchell-Olds 2003; Mitchell-Olds et al. 2007; Stinchcombe and Hoekstra 2007; Dalziel et al. 2009) has reinvigorated research into the genetics of adaptation. Below I briefly discuss a few potential avenues for future research on aspects of the genetics of adaptation that are still not well understood.

Adaptation to a moving optimum

The research described in this thesis focuses mainly on adaptive changes in marine populations of stickleback following introduction to freshwater. This is in accordance with the way that adaptation is typically studied; researchers investigate the changes that take place in a population after it is suddenly placed in an environment to which it is poorly adapted. However, few environmental changes other than those imposed by natural disasters occur instantaneously, and few natural environments remain constant over extended periods of time. The coastal lakes colonized by marine stickleback at the end of the last ice age would have made a gradual transition from marine to freshwater habitats as isostatic rebound isolated them from the ocean over a period of hundreds or

thousands of years (Bell and Foster 1994). Even climate change, which is expected to result in extremely rapid changes in selection regime, is not as drastic as the immediate change imposed in transplant experiments.

The discrepancy between the instantaneous environmental shifts used to study adaptation and the more gradual but continuous shifts expected in most natural situations may bias the inferences we draw from many studies of adaptive evolution. For instance, theory suggests that in conditions of slow environmental change, mutations with small effect tend to become fixed earlier than those with large effect (Collins et al. 2007; Kopp and Hermisson 2007). This is in direct contrast to what is found under constant selection after a sudden change in the environment (Orr 1998, 2002, 2003). It would be useful for future empirical studies of the genetics of adaptation to incorporate experimental designs that better represent the gradual nature of most environmental change. Given the logistical difficulties involved with this kind of environmental manipulation, it may be most realistic to employ a laboratory approach using microbial organisms in order to address this issue (e.g. Perron et al. 2008).

Epistatic effects on fitness

We have very few cases in wild populations in which a specific molecular change has been functionally associated with an ecologically significant trait (e.g. Hoekstra et al. 2006). Moreover, this level of resolution has only been achieved for single major effect genes whereas we know that most trait differences are governed by complex interactions between mutations at different genes. Thus, the gap in our knowledge of the molecular interactions responsible for ecologically relevant traits makes it difficult to generate

predictive models of adaptive evolution for most variation in nature (see Gratten et al. 2008). It is likely that epistasis between *Eda* and other minor effect loci plays an important role in the evolution of reduced armour in freshwater stickleback populations. An important avenue for future research into the genetics of adaptation will be to determine the genetic basis of a multigenic trait in a natural population. Once the genetic architecture of the trait has been determined, it would be informative to devise a way to directly measure the fitness consequences of specific mutations both individually and in combination. One possibility would be to create genetic lines carrying various combinations of alleles at loci implicated in a multigenic trait by making a series of crosses between divergent morphs. Each unique combination of alleles would represent the recreation of a possible step in the genetic trajectory leading to a morph, and therefore these genetic lines would provide a window into alternative evolutionary histories. Ideally, the fitness of the lines would then be measured under natural conditions, thus enabling estimation of the real world fitness consequences of interactions between mutations. Determining how interacting mutations are favored by natural selection, and the mechanisms by which they lead to adaptation, would provide insight into the genetic basis of morphological variation and the role of epistasis in adaptive change (e.g. de Visser et al. 2009)

The role of adaptation in the genetics of speciation

As stated in the introduction, Darwin felt quite strongly that adaptation played an important role in the creation of new species. He viewed species as groups of individuals that closely resembled each other, in which case adaptive divergence in phenotype leads

directly to speciation. The criterion for speciation shifted to reproductive isolation in the 1930s and 40s, but in the intervening years evolutionary biologists have accumulated a significant amount of data to show that this reproductive isolation often evolves as a result of local adaptation. The question is therefore no longer whether adaptation plays a role in speciation (i.e. does 'ecological speciation' occur?), but rather, what are the genetics of this process? Although there are many examples of divergent natural selection leading to reproductive isolation, there is typically no accompanying knowledge of the genes responsible (Schluter and Conte 2009). A useful way forward will be to use genetic mapping to identify the genes that underlie adaptive traits and to determine the functional mechanisms through which molecular changes at these genes contribute to the behavioural, mechanical, chemical and physiological incompatibilities that result in reproductive isolation. Furthermore, because speciation is a continuous process, it will be important to conduct this work in populations at various stages of adaptive divergence and to investigate the genetic changes contributing to multiple reproductive barriers.

The genetics of adaptation in stickleback

The majority of work described in this thesis has focused on testing functional mechanisms responsible for local adaptation in threespine stickleback. I have shown that parallel adaptive evolution in freshwater stickleback populations can be explained by the ecologically dependent and ontogenetically variable effects that standing genetic variation has on a number of morphological, behavioural and physiological traits. My results also provide evidence of how rapidly adaptation can occur when selection is strong and there is sufficient genetic variation in relevant traits. As is often the case with

empirical work, these experiments have raised as many questions as they have answered. Below I highlight some outstanding issues arising from these studies.

The heterozygote mystery

The changing fate of heterozygotes observed in Chapter 3 remains one of the most puzzling results in this thesis. While strong selection on *Eda* was observed during the experiment, the relative fitness of the three genotypes was equal when averaged over the full year. This suggests that for the low allele to fix in freshwater environments, a more complicated process must occur than simply a growth advantage leading to positive selection on low-plated adults. Because strong selection occurs prior to full lateral plate development, in Chapter 3 I suggest that variation at the *Eda* gene has direct or epistatic effects on other phenotypic traits contributing to fitness, or it is linked to another, unidentified locus affecting fitness. A major assumption of this work is that the mapping studies that implicated *Eda* for armor loss can be extended to the Oyster Lagoon source population for these experiments. The 2005 study by Colosimo and colleagues examined the results of just one cross and one polymorphic population, and inferences were extended to sticklebacks throughout the world using genealogical arguments (Colosimo et al. 2005). While it is possible that *Eda* alone is causative everywhere, and is the primary focus of selection, the possibility also exists that other genes in linkage disequilbrium with *Eda* are also involved with lateral plate loss and under selection. Genes in strong linkage disequilibrium would give a very similar phylogenetic pattern as that seen around *Eda*.

A common theme arising from fine mapping of QTLs is the presence of several tightly linked genes contributing to a trait in what was initially considered to be a single QTL (reviewed in Mackay et al. 2009). The linkage group where *Eda* resides is inherited in large blocks in some crosses, even over very large physical distances on the chromosome (Miller et al. 2007). Thus, the possibility exists that several linked loci are under selection after invasion of freshwater habitats by marine stickleback. This argument makes particular sense given the observed changes in heterozygote frequencies (Chapter 3). The data might be explained through epistatic effects among loci in this region that reduce fitness when the genomic region contains both 'freshwater' and 'marine' complements.

If the *Eda* low allele must be surrounded by other freshwater alleles in order to be free of deleterious epistatic effects in early development, then fixation of the allele following colonization of a new freshwater environment will be delayed until a sufficient number of rounds of recombination have brought freshwater alleles into the same genetic background. It has been suggested that adaptation to freshwater in stickleback occurs through a repeated process of selection on standing variation at numerous loci through a "transporter" process (Schluter and Conte 2009). Under this scenario, older freshwater populations provide the source of standing variation in the ancestral marine population. Alleles from freshwater adapted populations are exported to the ocean through hybridization between marine and stream populations. Multiple generations of recombination in the ocean then cause the freshwater adapted haplotype to be broken down. Eventually, marine individuals carrying small fragments of the freshwater haplotype colonize a new freshwater environment (perhaps created by a receding glacier).

Positive selection and recombination can then reassemble the separated freshwater alleles in the same genetic background. Thus, depending on how fractured the freshwater haplotype became during its sojourn in the ocean, the *Eda* low allele may re-enter freshwater relatively free of deleterious epistatic effects and be favoured by selection immediately, or alternatively there may be a long waiting time before the allele is surrounded by complementary freshwater alleles and can be positively selected.

In order to gain some understanding of the size of the freshwater haplotype typically surrounding *Eda* low alleles in the ocean, I am currently investigating the genetic differentiation between marine fish with homozygous low versus homozygous complete genotypes at microsatellite markers in neighbouring regions of the genome around *Eda*. I will compare this level of genetic differentiation with what is found between marine fish with homozygous complete genotypes and freshwater fish with homozygous low genotypes. By definition, genetic differentiation at *Eda* will be maximized in both cases. However, the expectation is that differentiation should remain high at other markers in the marine complete vs. freshwater low case, whereas differentiation should be reduced with increasing genetic distance from *Eda* in the marine complete vs. marine low case. The rate at which genetic differentiation drops with increasing distance from *Eda* will depend on a number of factors, including the strength of selection against freshwater alleles in the ocean, the amount of recombination in the genetic region including the freshwater haplotype, the level of gene flow between environments, and the amount of time since the low allele left freshwater. The use of fine scale genomic techniques, such as high-density single nucleotide polymorphism (SNP) arrays or restriction site associated DNA (RAD) markers may help to distinguish between

these mechanisms by providing the resolution to discriminate between the effects of genes in extremely close proximity.

The genetics of temperature tolerance in stickleback

In Chapter 7, I presented evidence of extremely rapid phenotypic evolution of cold tolerance in marine stickleback transplanted to freshwater. While climate change is predicted to lead to an increase in both hot and cold temperature extremes, the main direction of temperature change is expected to be towards warmer average temperatures (Meehl and Tebaldi 2004; Schar et al. 2004; Lenton et al. 2008; Ramanathan and Feng 2008). To be able to predict the potential success or failure of stickleback populations from around the world to adapt to increased temperatures it will be crucial to identify the genes responsible for temperature tolerance. This will allow us to determine which populations possess sufficient genetic variation to evolve in response to the selection imposed by climate change. Identifying the genes that are responsible for temperature tolerance will be facilitated by studying populations from the extreme latitudes inhabited by the species, which are likely to possess greater genetic variation in this trait. Sticklebacks have a broad distribution in marine and freshwater habitats that extends from Mexico to Alaska. Importantly, these geographically distant populations may express variation in heat tolerance. This variation was not detected in the populations sampled in British Columbia, but may be a crucial for permitting adaptation to climate change. Latitudinal gradients are ideal systems to study the genetic mechanisms for coping with environmental variation in nature (Powers and Schulte 1998; Balanya et al. 2006). Studies of adaptation to clinal variation have provided insight into the ways that

genetic variation enables organisms to cope with rapid environmental change, including temperature adaptation (Umina et al. 2005) and photoperiodism (Bradshaw and Holzapfel 2008). However, these studies have employed a "bottom-up" genetics approach, targeting specific genes and investigating their effect on phenotype. This approach risks missing relevant genes and may impede explanations of adaptation when traits co-evolve together. In contrast, a "top-down" genetics approach avoids this problem by starting with the phenotype that confers improved environmental tolerance and then determining which genes are responsible for the trait (through QTL analysis and other approaches). In the future I will collaborate with others to use a "top-down" approach to identify the genetic mechanisms responsible for adaptive temperature tolerance in stickleback.

Concluding statement

We have learned much about the process of adaptation since Darwin first postulated that it plays a central role in evolution. In the 1880s, after correspondence with Darwin, Reverend W.H. Dallinger conducted what was probably the first selection experiment. Dallinger wanted to determine experimentally "whether it was possible by change of environment… to superinduce changes of an adaptive character, if the observations extended over a sufficiently long period." He reared protists under gradually increasing temperatures over a period of seven years. Before the experiment was ended by an accident, the protists were able to tolerate temperatures far in excess of their ancestors. This provided the first hard experimental support for evolution by natural selection. In the ensuing decades considerable progress was made in quantifying selection on phenotypic traits in natural populations. However, it was not until almost a full century after

Dallinger's study that a new experimental approach made it possible to collect rigorous data about the causal genes underlying the adaptation. Genetic mapping of quantitative trait loci has helped to identify the genetic regions responsible for phenotypic differences between populations or species. As genomic technologies and methodology have advanced in recent years, the level of resolution has improved to enable discrimination of the molecular changes in these candidate regions or genes. Knowing the identity of the specific genetic changes involved in adaptive evolution will make it possible to answer a number of fundamental questions that were previously out of reach when quantitative genetics remained under a "black box":

1. How do mutations interact to generate ecologically relevant variation?
2. Is the effect of mutations occurring early in the adaptive process greater than the effect of mutations occurring late in the adaptive process?
3. Does adaptation to similar environments usually involve the same genes and mutations, and is the pattern and pace of their use the same over evolutionary time?
4. Are mutations used in adaptation typically recessive or dominant, coding or regulatory, large effect or small effect, pre-existing or *de novo*?

Knowledge of the genes responsible for adaptive phenotypes is also desirable for the sake of intellectual completeness. This is because each level of biological organization finds its explanations of mechanism in the levels below it and its significance in the levels above it. As demonstrated by the experiments described in this thesis, it is now possible to test the functional mechanisms that give a gene ecological importance. The synthesis of knowledge from different levels of biological organization is essential to understanding the patterns of diversity we see in nature.

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APPENDICES

Appendix A – Supplementary figure for chapter 3

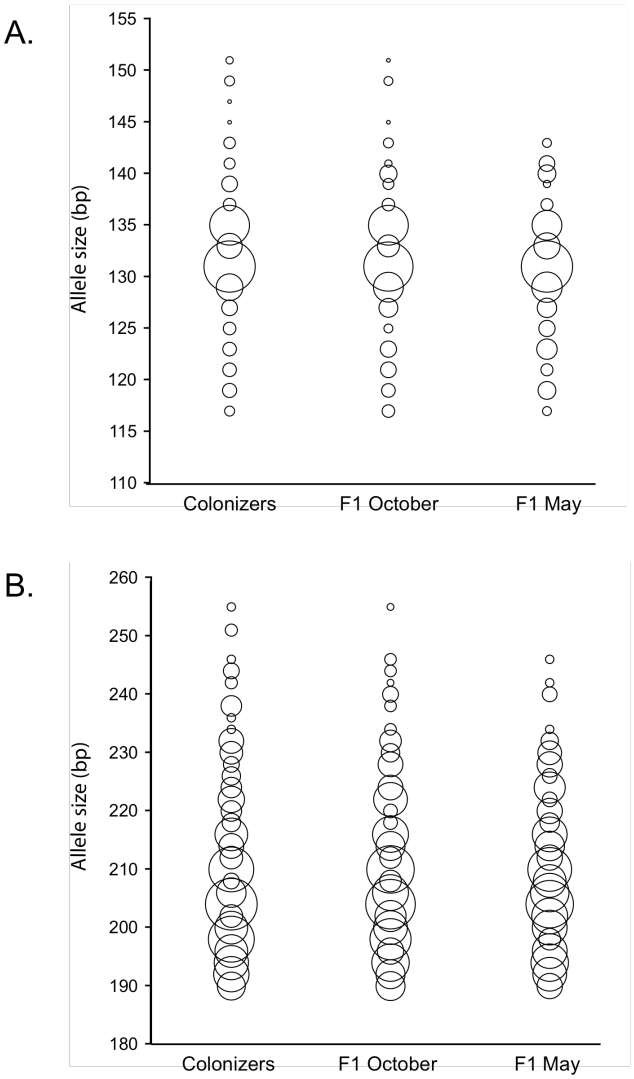

Figure A.2. Frequency distribution of alleles at two neutral microsatellite markers unlinked to Eda*, Stn224 on linkage group 11 (A) and Stn 314 on linkage group 8 (B).*

Each circle along the vertical axis indicates a distinct allele (in base pair size) while the diameter of each circle is proportional to the relative allele frequency calculated for all ponds combined. An analysis of the allele frequencies at both loci with Fisher's combined probability test indicated that both the colonizers ($\chi^2 = 2.73$, $df = 4$, $P = 0.60$) and the individuals sampled in October 2006 ($\chi^2 = 8.06$, $df = 4$, $P = 0.09$) and May 2007 ($\chi^2 = 7.05$, $df = 4$, $P = 0.13$) were in Hardy-Weinberg Equilibrium.

Appendix B – Supplementary table for chapter 4

Table B.1. Association between Eda genotype and armor phenotype

		Armor phenotype		
Salinity treatment	*Eda* genotype	Complete	Partial	Low
Salt	CC	47	2	0
	CL	11	30	1
	LL	1	19	15
Fresh	CC	51	3	0
	CL	43	31	2
	LL	1	24	32

Salt: $\chi^2 = 105.93$, d.f. = 4, $p < 10^{-15}$, n = 126. Fresh: $\chi^2 = 128.72$, d.f. = 4, $p < 10^{-15}$, n = 187. Individuals pooled from all blocks. There were no significant treatment effects on genotype-phenotype associations (heterogeneity $\chi^2 = 8.1$, d.f. = 4, p = 0.09). *Eda* genotypes are based on the Stn381 in/del marker: "complete" (C) alleles represent 162 or 171 bp bands, and "low" (L) alleles represent 191 bp bands.

Appendix C – Supplementary material for chapter 7

Table C.1. Temperature monitoring locations

Type	Location	Coordinates
Marine	Active pass	48°52’00"N 123°17’00"W
Marine	Amphitrite point	48°55’00"N 125°32’00"W
Marine	Bonilla Island	53°30’00"N 130°38’00"W
Marine	Chrome Island	49°28’00"N 124°41’00"W
Marine	Departure Bay	49°13’00"N 123°57’00"W
Marine	Egg Island	51°15’00"N 127°50’00"W
Marine	Entrance Island	49°13’00"N 123°48’00"W
Marine	Kains Island	50°27’00"N 128°02’00"W
Marine	Langara Island	54°15’00"N 133°03’00"W
Marine	McInnes Island	52°16’00"N 128°43’00"W
Marine	Halibut Bank	49°30’00"N 123°70’00"W
Freshwater	Okanagan	52°07'02"N 122°04'22"W
Freshwater	St Mary's	48°53'26"N 123°32'34"W
Freshwater	Cusheon	48°48'55"N 123°28'02"W
Freshwater	Kalamalka	50°10'04"N 119°20'31"W
Freshwater	Shuswap	50°56'00"N 119°16'60"W
Freshwater	Mara	50°47'13"N 119°00'20"W
Freshwater	Mabel	50°32'60"N 118°44'11"W
Freshwater	Sugar	50°23'50"N 118°30'58"W
Freshwater	Skaha	49°24'51"N 119°35'03"W
Freshwater	Long	49°54'57"N 125°28'13"W
Freshwater	Quinsam	49°52'24"N 125°33'27"W
Freshwater	Williams	52°07'02"N 122°04'22"W
Freshwater	Osoyoos	49°01'13"N 119°27'22"W
Freshwater	Quenell	49°04'13"N 123°48'31"W

Table C.2. Sampling locations

Type	Location	Area	Coordinates
Marine	Little Campbell	Semiahmoo First Nation Territory	49°00’58”N 122°46’44W
Marine	Oyster Lagoon	Sechelt Peninsula	49°36’48”N 124°01’47”W
Freshwater	Cranby Lake	Texada Island	49°41'45"N 124°30'28"W
Freshwater	Hoggan Lake	Gabriola Island	49°09'08"N 123°49"W

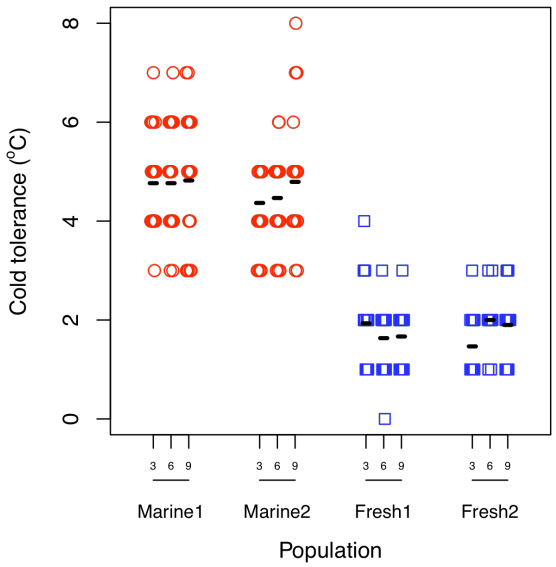

Figure C.1. Cold tolerance trials over different acclimation periods.

Red circles and blue squares indicate individual fish from marine and freshwater populations, respectively. Each individual was tested following three, six and nine weeks of acclimation to the lab. Black bars indicate mean values. Marine1 = Little Campbell, Marine2 = Oyster Lagoon, Fresh1 = Cranby Lake, Fresh2 = Hoggan Lake.

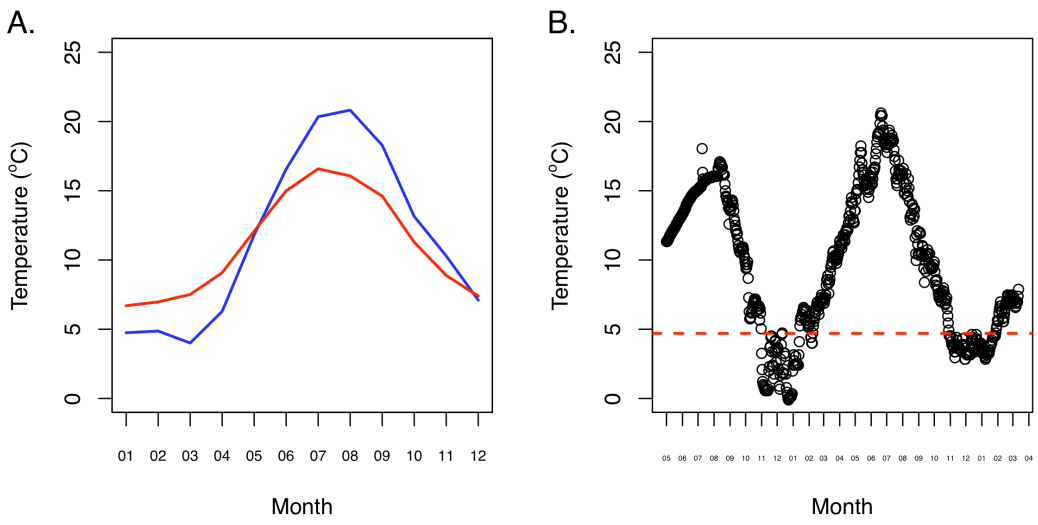

Figure C.2. Water temperature data from British Columbia, Canada.

(A) Monthly mean temperature averaged from 11 marine sites (red) and 14 freshwater lakes (blue) in British Columbia (See Table C.2 for locations). (B) Daily minimum temperature averaged from 3 ponds located at the University of British Columbia, Vancouver, British Columbia between May 2006 and May 2008. Dashed line shows the minimum temperature in Oyster Lagoon, British Columbia.

Appendix D – Supplementary material for chapter 8

The effect of fluctuating population size on fixation probability and the time to fixation

Assuming weak selection and a fluctuating population size, the average probability of fixation of a beneficial mutation is approximately $2sN_e/\overline{N}$ (EWENS 1967; KIMURA and OHTA 1974; OTTO and WHITLOCK 1997). Here, the arithmetic average population size is $\overline{N}$, and the "effective" population size is N_e, whose calculation depends on the nature of the population fluctuations (OTTO and WHITLOCK 1997; WAHL *et al.* 2002). Unfortunately, we lack an analytical expression for the fixation probability when selection is strong and population size varies. We conjecture that an adequate approximation for the average fixation probability under strong selection is given by $\overline{P} = 1 - e^{-2sN_e/\overline{N}}$, which is nearly $2sN_e/\overline{N}$ when selection is weak but has the advantage of remaining less than one when selection is strong. This approximation is equivalent to the one used when the population size is constant, i.e., $N_e = \overline{N}$ (see Figure 8.1). This functional form is also suggested by diffusion analysis in populations of large effective size (KIMURA 1957; KIMURA 1964), which assumes weak selection. Simulations confirm that $\overline{P}$ provides a satisfactory approximation for the fixation probability over a range of parameter values in populations undergoing repeated bottlenecks (within a factor of two; Figure D.3).

We next consider the time to fixation of a beneficial mutation. If the mutation arises when the population size is N_a and fixes when the population size is N_f, a deterministic model of selection can again be used to predict that:

$$T = \ln\left(\left(N_a - 1\right)\left(N_f - 1\right)\right)/\ln\left(1 + s\right) \approx \ln\left(N_a N_f\right)/\ln\left(1 + s\right).$$

Mutations are more likely to arise when the population size is large, but they are more likely to fix when the population size is small. Averaging the time to fixation over all possible events requires precise knowledge of the fluctuations in population size and the strength of selection. Assuming that mutations arise and fix uniformly over time, however, provides a generic approximation for the time to fixation:

$$\begin{aligned} \overline{T} &\approx \sum_{f=1}^{\tau} \sum_{a=1}^{\tau} \frac{\ln\left(N_a N_f\right)}{\tau^2 \ln(1+s)} \\ &= \frac{2\ln\left(N_{gm}\right)}{\ln(1+s)} \end{aligned} \tag{9}$$

where N_{gm} is the geometric mean population size over time. (The first line in equation 9, but not the second, assumes that the population size cycles over time with period τ.) Simulations indicate that $\overline{T}$ provides a satisfactory approximation for the average time to fixation over a range of parameter values in populations undergoing repeated bottlenecks (within a factor of two; Figure D.4).

To account for clonal interference, we should determine the expected number of mutations that compete for fixation when the focal mutation appears at time *t* (see equation 5) and then average over all possible times at which the focal mutation could arise. To do so exactly requires a precise description of the manner in which the population size fluctuates. As a first order approximation, we estimate the number of competing mutations using:

$$\overline{\lambda}(s) = \overline{P}\ \overline{N}\ \mu \frac{\overline{T}}{2} \tag{10}$$

This approximation ignores the covariance between the number of contending mutations and the time to fixation of a focal mutation, which should be generated by the fluctuations in population size.

Using equation (10) in (7), the cdf among fixed beneficial mutations becomes:

$$H(s) = N_{gm}^{-\overline{N}\,\mu\,\kappa/\ln(1+s)}\left(1-\frac{\kappa}{\Pi}\right) \tag{11}$$

where κ is again given by equation (8) and the average probability of fixation across the distribution of new mutations is now:

$$\Pi = 1-\left(1+2\,\sigma\,cv^2\,\frac{N_e}{\overline{N}}\right)^{-1/cv^2} \tag{12}$$

The corresponding probability density function for fixed mutations is $h(s) = \mathrm{d}H(s)/\mathrm{d}s$.

We assessed the accuracy of equation (11) against simulations of a population whose size cycles from N_0 to $2^7\,N_0$ via seven doubling events followed by a $1/2^7$ serial dilution. In these simulations, the growth of the population was assumed to be deterministic (no sampling except during the dilution or "bottleneck" generation), and births occurred at a rate proportional to the fitness of an individual. Under this scenario, the size of the bottleneck, N_0, and the period of the cycle, τ, determine $N_e \approx N_0 \ln(2)\tau$ (WAHL *et al.* 2002), $\overline{N} \approx N_0\left(2^\tau - 1\right)/\tau$, and $N_{gm} \approx N_0\sqrt{2^{\tau-1}}$ for use in equation (11). Every combination of the following parameters was explored: selection coefficients (σ: 0.01, 0.1, 1, 2, 10), beneficial mutation rate ($\mu = 10^{-7}, 10^{-9}$), and initial population size ($N_0 = 10^5, 10^6, 10^7$), assuming that the fitness effects of new mutants was exponential ($cv = 1$).

Figure 8.5 indicates that equation (11) accurately predicts the distribution of fixed selective effects across this range of parameters. Interestingly, equation (2) of Rozen et al. (2002) provides a more accurate prediction of the distribution of fixed beneficial mutations with a fluctuating population size (with N_e in place of N) than with a constant population size (Figure 8.2). The improved performance of their method is due to the fact that the fixation probability used, $2sN_e/\overline{N}$, remains reasonably accurate even when selection is strong ($s >> 0.1$) because of the reduction in effective population size caused by the fluctuations ($N_e << \overline{N}$).

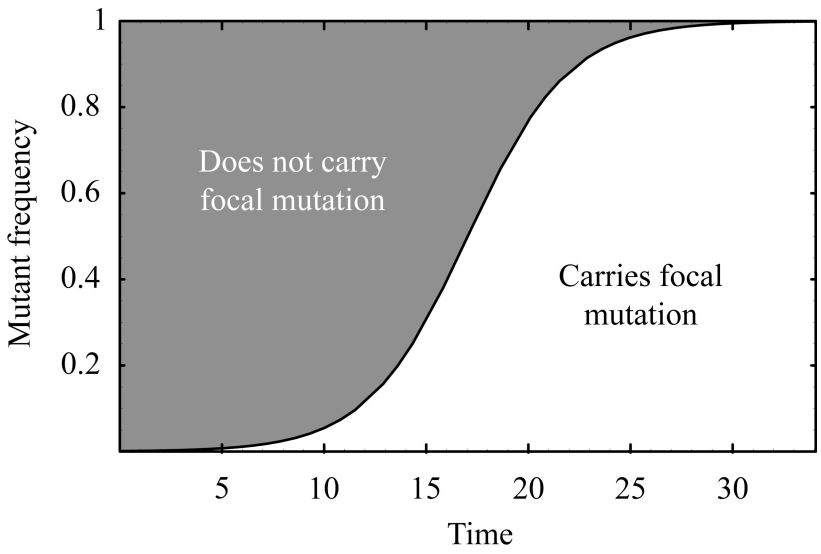

Figure D.1. The spread of a beneficial mutation from one copy (p0 = 1/N) to fixation (p > 1 – 1/N) is described by a symmetrical S-shaped curve in haploid populations.

The area above the curve (shaded) represents the proportion of the population that does not carry the focal mutation. When averaged across the entire time to fixation, this shaded area equals 1/2 of the total area (given by $1 \times T$, the time to fixation).

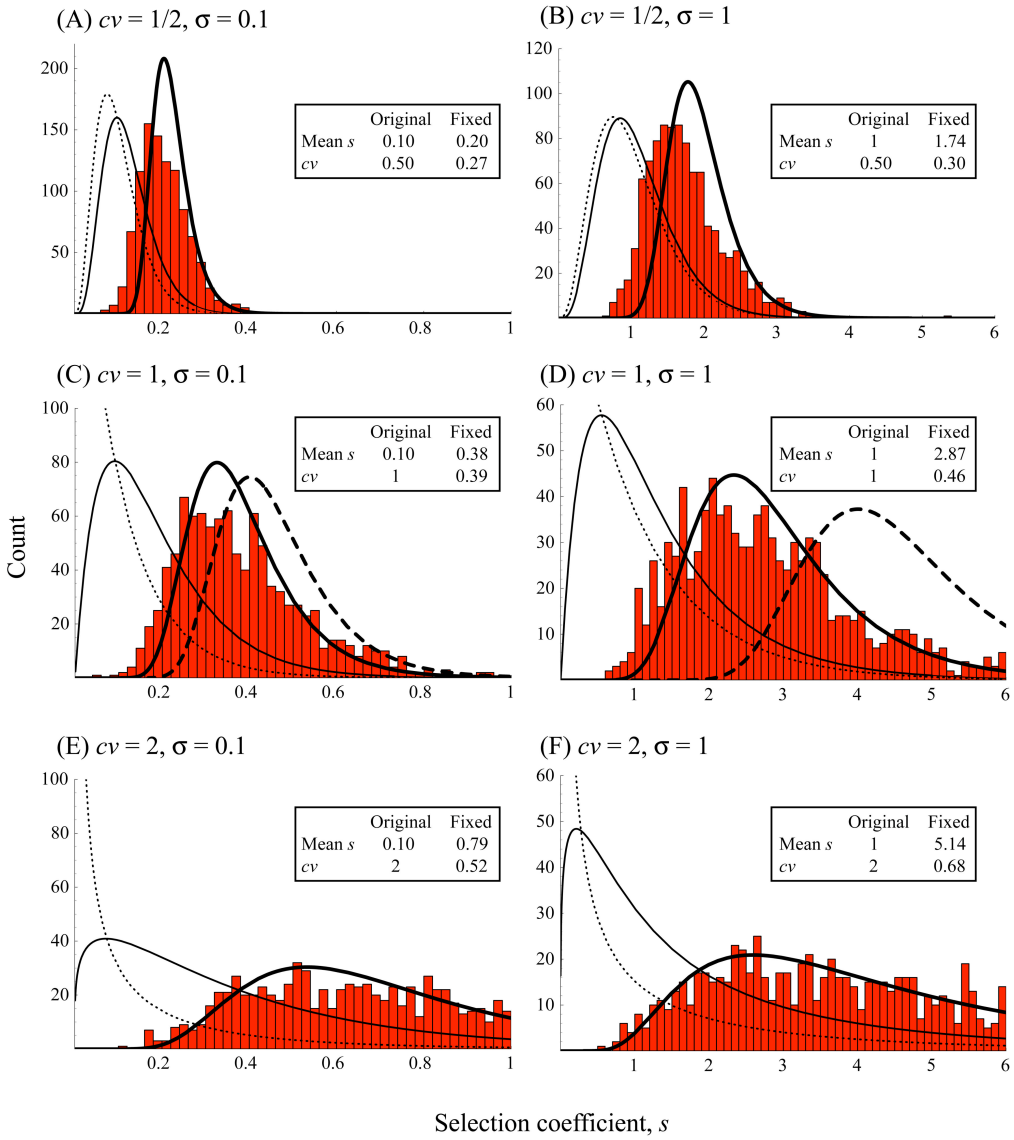

Figure D.2. Effect of the shape of the original distribution on fixed mutations.

New mutations were distributed according to a gamma distribution, $f(s)$ (thin dotted curve). Contending mutations that survived loss while rare (thin solid curve) are shifted to the right ($g(s)$ from equation 4). Fixed mutations that also survived clonal interference are shifted even further to the right (histogram: simulations; thick solid curve: our equation 7; thick dashed curve: equation 2 of Rozen et al. (2002) available only for cv = 1). The coefficient of variation among newly arising mutations is lowest in the top panels (cv = 1/2), intermediate in the central panels (cv = 1; identical to panels C and D in Figure 8.3), and highest in the bottom panels (cv = 2). The average strength of selection increases from the left panels (σ = 0.1) to the right panels (σ = 1). Remaining parameters: $\mu = 10^{-5}$ and $N = 10^5$.

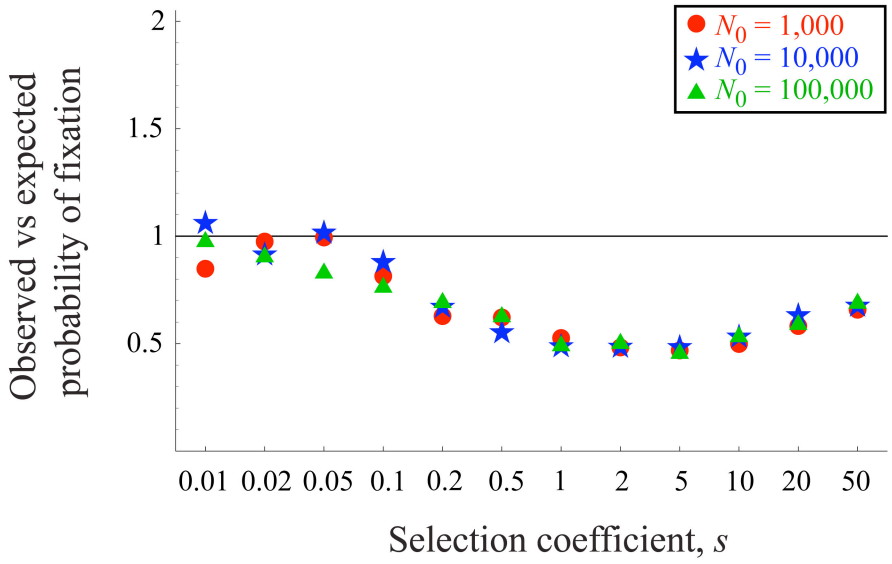

Figure D.3. The average probability of fixation of a beneficial mutation observed in simulations with a fluctuating population size, divided by the expected value, $\overline{P} = 1 - e^{-2sN_e/\overline{N}}$.

This probability of fixation performed much better than the commonly used approximation, $2sN_e/\overline{N}$, when selection was strong (data not shown). The population doubled in size each generation from size N_0 to $2^7 N_0$, followed by a bottleneck back to size N_0. A single mutation was introduced at a random point in the cycle, in proportion to the population size at that time. No contending mutations were allowed. Simulations were replicated until 200 fixation events were observed.

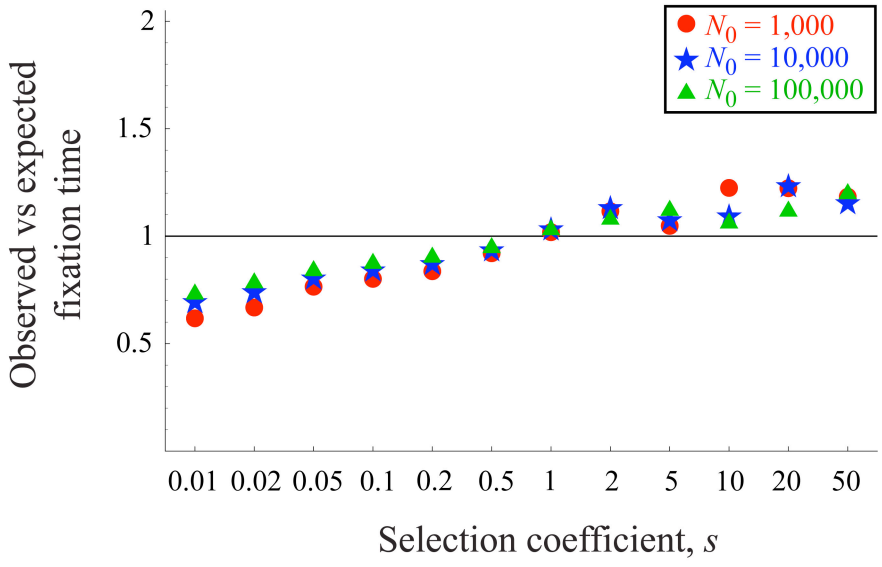

Figure D.4. The average time to fixation of a beneficial mutation observed in simulations with a fluctuating population size, divided by the expected value, $\overline{T} = 2\ln(N_{gm})/\ln(1+s)$. When selection was strong, this expected value performed much better than the weak selection approximation, $\overline{T} = 2\ln(N_e)/s$ of Rozen *et al.* 2002 (data not shown). The population doubled in size each generation from size N_0 to 2^7 N_0, followed by a bottleneck back to size N_0. A single mutation was introduced at a random point in the cycle, in proportion to the population size at that time. No contending mutations were allowed. Simulations were replicated until 200 fixation events were observed.

Appendix E – Animal care permits

2006 Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A04-0208

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:

Brienomyrus magnostipes 500
Fish 1000

Start Date: April 28, 2005 **Approval Date:** July 21, 2006

Funding Sources:

Animal Protocol Number: A04-0208
Grant Agency: Centre National De La Recherche Scientifique
Grant Title: Resting the predictions of ecological speciation in sticklebacks (EcoSpec)
Grant Number: 04-5521

Grant Agency: Natural Science Engineering Research Council
Grant Title: Ecology, genetics, and the origin of species
Grant Number: 00-3385

Grant Agency: BC Ministry of Environment, Lands and Parks
Grant Title: Population Assessment for Stickleback Species Pairs
Grant Number: 06-0798

Animal Protocol Number: A04-0208
Grant Agency: Natural Science Engineering Research Council
Grant Title: Ecology, Genetics and the Origin of Species

Grant Agency: Natural Science Engineering Research Council
Grant Title: Ecology and genetics of adaptive radiation

Grant Agency: Natural Science Engineering Research Council
Grant Title: The Genetics of Adaptation to New Environments

Grant Agency: National Geographic Society
Grant Title: National Geographic grant to collect electric fishes in Gabon

Grant Agency: Royal Society of Canada
Grant Title: Ecological Causes of Adaptive Radiation

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

2007 Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0293

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:

Trout	Cutthroat trout 10
Fish	Prickly sculpin 100
Sticklebacks	Threespine stickleback 4000

Start Date: April 1, 2006 **Approval Date:** September 17, 2007

Funding Sources:

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Unfunded title: Ecology and genetics of adaptive radiation

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

2008 Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0293

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:

Sticklebacks	Threespine stickleback 4000
Invertebrates	Backswimmers (Notonecta) and dragonfly nymphs (Aeshna) 750
Trout	Cutthroat trout 10
Fish	Prickly sculpin 100

Start Date: April 1, 2006 **Approval Date:** November 17, 2008

Funding Sources:

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Funding Agency: National Institutes of Health
Funding Title: 1 F32 GM086125-01 Genetics of Behavioral Reproductive Isolation in Threespine Stickleback, Schluter/Arnegard

Unfunded title: Ecology and genetics of adaptive radiation

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration

2009 Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0293

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:

Fish	Prickly sculpin 100
	Trout Cutthroat trout 10
Invertebrates	Backswimmers (Notonecta) and dragonfly nymphs (Aeshna) 750
Sticklebacks	Threespine stickleback 4000

Start Date: April 1, 2006 **Approval Date:** December 10, 2009

Funding Sources:

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: National Institutes of Health
Funding Title: 1 F32 GM086125-01 Genetics of Behavioral Reproductive Isolation in Threespine Stickleback, Schluter/Arnegard

Unfunded title: Ecology and genetics of adaptive radiation

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
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