From invasion to diversification –
studying the speciation continuum in stickleback

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To Claudia, Mélissa and Nehla

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Introduction

At a time where biodiversity seems to decline at a constant pace (Hoffmann *et al.*, 2010), it becomes increasingly important to assess the actual ecologically and functionally relevant diversity that may lay cryptically hidden within previously defined species as well as to understand the evolutionary dynamics that underlie the emergence and maintenance of biodiversity (e.g. (Chevin *et al.*, 2010; Vonlanthen *et al.*, 2012). Anthropogenic alterations and perturbations of entire ecosystems account for most of the known threats to biodiversity, including global warming (Pereira *et al.*, 2010). Other examples come from anthropogenic environmental perturbation that change the adaptive landscapes leading to a rapid and massive loss of biodiversity (Seehausen *et al.*, 1997; Taylor *et al.*, 2006; Vonlanthen *et al.*, 2012) or the introduction of invasive species (Elton, 1958; Lockwood *et al.*, 2007). Such events on the other hand may serve as unintentional evolutionary experiments in the wild and provide unique opportunities to study many contemporary evolutionary processes, which applies especially for invasive species (Westley, 2011; Abbott *et al.*, 2013). Only over the last two decades theoretical links have been established between invasion biology and general evolutionary theory (Sakai *et al.*, 2001; Prentis *et al.*, 2008; Losos, 2010; Westley, 2011; Abbott *et al.*, 2013). Indeed similar evolutionary processes that underlie the process of speciation seem to operate during a successful invasion and subsequent diversification, hence leading to the emergence of biodiversity (Hendry *et al.*, 2000; Koskinen *et al.*, 2002; Abbott *et al.*, 2013). However, despite these recent advances only little is yet known about the relative importance of the different underlying evolutionary processes in empirical systems and how the interplay of these processes may finally promote speciation.

The aim of this thesis is to bridge the gap between the study of biological invasions and eco-evolutionary processes in the context of adaptive radiations and hence the emergence of new species and biodiversity. It further aims to contribute to the understanding of evolutionary processes that underlie adaptive diversification during a biological invasion, providing the opportunity to study the potential onset of adaptive radiations. It focuses on the evolutionary diversification of threespine stickleback (*Gasterosteus aculeatus*) – a fish species that has become a model system in evolutionary ecology due to its repeatedly and independently evolved ecotypes and species since the last glaciation period (McKinnon & Rundle, 2002), but which only became introduced in large parts of Switzerland about 140 years ago. Since then, sticklebacks have colonized ecologically very different habitats and have an increased phenotypic diversity than observed in their native range (Lucek *et al.*, 2010). Combining experimental, ecological, morphological, genetic and quantitative genetic approaches this thesis intents to draw a comprehensive picture of the eco-evolutionary aspects of this biological invasion in comparison to natural and evolutionary older stickleback populations.

Adaptive radiation

Adaptive radiation can be generally defined as the proliferation of a single ancestral lineage into a variety of species adapted to different ecological niches and is one of the most important processes in the origin of species diversity (Losos, 2010). Consequently the study of adaptive radiations is a key to understand the evolutionary processes that lead to the rise and maintenance of biodiversity. Indeed, adaptive radiations account for a great amount of the current biodiversity in many taxa, as for example in Hawaiian *Drosophila* (Kambysellis *et al.*, 1995) or the radiation of African cichlid fishes (Wagner *et al.*, 2012), each comprising cases with more than a thousand different species. In the Northern Hemisphere, many classical examples of adaptive radiations derive from fish species in postglacial lakes that evolved since the recolonization of freshwater bodies (Smith & Skúlason, 1996). Here, parallel cases of adaptive ecological diversification have been repeatedly documented as for example for whitefish *Coregonus sp.* (Hudson *et al.*, 2011), the threespine stickleback *Gasterosteus aculeatus* (McKinnon & Rundle, 2002) and in arctic charr *Salvelinus salvelinus* (Jonsson & Jonsson, 2001).

Adaptive radiation or not? A matter of definition.

The term "adaptive radiation" was originally coined by Osborn (1902) and defined by Simpson (1953) as the "more or less simultaneous divergence of numerous lines all from much the same ancestral adaptive type into different, also diverging adaptive zones", where the "progressive occupation of such zones is not simultaneous and usually involves in any one period of time the change of only one or a few lines from one zone to another, with each transition involving a distinctly different ancestral type" (Simpson, 1953; p. 223). Many examples for adaptive radiations have emerged since then, including Darwin finches (Grant, 1981), threespine stickleback (Schluter, 1993) or Anoles lizards (Losos, 2009). Almost five decades after Simpson, Schluter (2000) defined an adaptive radiation in his seminal book "*The Ecology of Adaptive Radiation*" similarly as the "evolution of ecological and phenotypic diversity within a rapidly multiplying lineage" with "the differentiation of a single ancestor into an array of species that inhabit a variety of environments and that differ in the morphological and physiological traits used to exploit those environments" (Schluter, 2000; p. 10). Yet he defined four prerequisites that describe an adaptive radiation and which have since been commonly applied: 1) a common ancestry of each species within a radiation, 2) a significant relationship between the occupied environments and the phenotypic traits that are used to exploit them, 3) trait utility, i.e. to proof that specific traits provide a fitness advantages in their corresponding environments, 4) rapid speciation events, i.e. the sudden burst of species during ecological and phenotypic divergence. This definition relies however on the fact that the process of speciation has already advanced, leading to genetically and phenotypically distinct species, where adaptive phenotypes are mainly genetically determined. Consequently many potential cases of adaptive radiations fail to fulfill one or several of the prerequisites defined by Schluter (2000) either due to biological constraints, such as in bacteria, where the delimitation of species is not resolved and horizontal gene flow is common

(Kassen, 2009) or because the studied systems are evolutionary very young. The latter case is especially true in postglacial radiations of freshwater fishes. Here speciation is commonly incomplete and adaptive plasticity in combination with selection on standing genetic variation can lead to the emergence of distinctly adapted ecotypes in many cases (Smith & Skúlason, 1996; Hendry *et al.*, 2009), but see (Jones *et al.*, 2012a). Because such radiations form nevertheless stable and ecological distinct population that may even be reproductively isolated, a relaxed definition for adaptive radiation is needed (Losos, 2010) that emphasizes on the functional phenotypic aspects of adaptive radiations. An example for such a definition comes from Futuyma (1997), who defines an adaptive radiation as the "evolutionary divergence of members of a single phylogenetic line into a variety of different adaptive forms; usually the taxa differ in the use of resources or habitats, and have diverged over a relatively short interval of geological time". The advantage of such phenotype based definitions is that potential radiations can be identified from phenotypically and ecologically divergent populations, independent of the underlying evolutionary processes, e.g. in recently diverged systems (Sandlund, et al., 1992a) or fossils (Neubauer *et al.*, 2012). In such cases, adaptivity of the distinct phenotypes needs to be inferred in order to distinguish adaptive from non-adaptive radiations (Schluter, 2000), either directly through experiments (e.g. Lundsgaard-Hansen *et al.*, 2013) or indirectly through comparisons with similarly diverged species (see Chapter 5 for an example).

The emergence of adaptive radiation

The emergence of an adaptive radiation and its progression can be characterized by different stages, spanning from the colonization of a novel environment by a single species over its subsequently diversification into ecologically distinct ecotypes and species to a complete radiation, where all available niches are filled up by reproductively isolated species. At each stage of this continuum, different evolutionary processes may operate (Simpson, 1953; Schluter, 2000). This leads to distinct patterns that emerge through time on both the phenotypic and genotypic level affecting as well their related quantitative genetic structure (Figure 1).

The very beginning of an adaptive radiation is characterized by the colonization of a new and potentially competition free environment by a single species (Simpson, 1953; Schluter, 2000). However, not all such colonization events succeed to form adaptive radiations (Losos, 2010; Wagner *et al.*, 2012). The intrinsic factors that either promote or impede a successful establishment and colonization are similar to the ones at play during biological invasion (Losos, 2010; Yoder *et al.*, 2010), which are outlined in detail in the sections below. In short, a species may need to overcome different obstacles, depending on its ability to cope with a novel environment, such as overcoming former genetic constraints due to founder effects or the lack of adaptive genetic variation. Such constrains may be resolved through hybridization, generating novel phenotypes that allow to explore a wider niche space (Seehausen, 2004; Stelkens *et al.*, 2009). Similarly the evolution of "key innovations" that let a species to interact with the environment in a novel way have been suggested to promote adaptive radiations

(Simpson, 1953; Schluter, 2000; Gavrilets & Losos, 2009). In addition, phenotypic plasticity can promote the initiation of adaptive radiation, allowing a species to express rapidly adapted phenotypes (West-Eberhard, 2003). Being potentially beneficial at an early stage, phenotypic plasticity may conversely hamper successful diversification at a later stage by shielding the genome from selection (Ghalambor *et al.*, 2007; Lande, 2009; Thibert-Plante & Hendry, 2011, see below).

From its original point of colonization, the founding species will undergo a range expansion and spread throughout the available environment, where it faces different selection pressures that are related to different parts of the available niche space. Such a heterogeneous adaptive landscape imposes divergent natural selection on individuals across the newly colonized range (Figure 1c), selecting for local adaptation and differentiation between ecologically distinct parts of the colonized range (Gavrilets, 2004; Gavrilets & Losos, 2009). Subsequent adaptation and specialization may lead to the emergence of reproductive isolation and finally to the formation of ecologically distinct species (Schluter, 2009; Nosil, 2012). By filling the available niche space, the number of evolved species is expected to increase through time. However, a radiation may reach a species overshoot or a plateau, were the speciation rate is decreasing and/or the extinction rate is increasing (Schluter, 2000; Gavrilets & Losos, 2009). Overshooting may occur if different species start to fill similar niches, leading to strong interspecific competition and further extinction (Simpson, 1953; Gavrilets & Vose, 2005) or because less niches are available over time, decreasing the propensity for speciation. Theoretical (Gavrilets & Vose, 2005) and empirical (Schluter, 2000; Gillespie, 2004) evidence for a species overshoot is however limited. Throughout the adaptive radiation process, the phenology of the processes driving phenotypic, genotypic and the related quantitative genetic patterns differ:

Adaptive diversification commonly leads to phenotypic differentiation in ecologically relevant traits between individuals occupying different ecological niches and thereby experiencing divergent selection. This leads to the occurrence of distinct phenotypic clusters (Leimar *et al.*, 2008; Nosil, 2012), associated with ecological factors, where selection leads to a correlation between a trait and its selective environment (Schluter, 2000; Nosil, 2012). The overall phenotypic variation across the whole radiation comprising all such phenotypic clusters should consequently increase over time as more and more ecotypes and species emerge (Gavrilets & Losos, 2009, Figure 1a). Filling up formerly unused niche space or by creating additional new niches themselves e.g. by creating new links in the food chain, such as the evolution of predatory species, may further fuel the increase in overall phenotypic diversity and lead to a burst-like pattern of phenotypic variation (Schluter, 2000; Gavrilets & Losos, 2009). Phenotypic variation may however peak or even decrease over time as fewer niches become available and the extinction rate similar to the rate of speciation (Schluter, 2000; Gavrilets & Losos, 2009) as has been found to be the case in many fossil groups (Foote, 1993). Such declines may however occur only over relatively large time spans and seem to be absent in more recent groups (Schluter, 2000). Hence, overall phenotypic variation may either decrease or an equilibrium is reached,

where further phenotypic variation could slowly emerge over time by random processes.

Genetic variation on the other hand may initially undergo a decrease in the colonizing population in comparison to its ancestor due to potential founder effects and genetic bottlenecks (Dlugosch & Parker, 2008) or because only a fraction of the ancestral standing genetic variation may have become selected (Barrett & Schluter, 2008). Likewise during the range and niche expansion phase, bottlenecks, founder effects and drift may further decrease the genetic variation of a population at the colonization perimeter. The levels of standing genetic variation may moreover depend on the initial population size of the colonizer and its degree of standing genetic variation (Barrett & Schluter, 2008; Simberloff, 2009). In addition, introgression and hybridization may initially increase the levels of standing genetic variation (Seehausen, 2004). Neutral and to a lesser extent adaptive genetic variation may otherwise increase slowly through mutation, where local adaptation and further diversification may require time to gain the adaptive genetic potential needed. This may be similar to observed lag phases in many invasive species that are confronted to a new environment, where introduced populations persist only locally with a relatively small population size before they undergo a range expansion (Sakai *et al.*, 2001; Dlugosch & Parker, 2008). Genetic differentiation among diversifying populations should analogously increase through time (Thibert-Plante & Hendry, 2010, Figure 1c) as ecologically distinct populations become increasingly isolated and finally forming distinct species (Feder *et al.*, 2012). Initial genetic differentiation may occur in only few loci, which underlie parts of the genome that experience divergent selection, forming "islands of selection" (Feder *et al.*, 2012; Nosil, 2012). Gene flow may then become increasingly restricted around such islands and eventually connecting them, resulting in an increased genomic differentiation – or genetic "continents of selection" – among ecological distinct species (Nosil, 2012).

Evolutionary responses during an adaptive radiation may underlie genetic constraints due to genetic covariation (Schluter, 1996). Consequently the direction of evolution should be predictable using quantitative genetic models (Lande, 1979; Lande & Arnold, 1983, see below). Indeed, empirical evidence suggest that adaptive radiation may evolve initially among similar evolutionary trajectories – or so called lines of least resistance (Schluter, 1996). Subsequent diversifying selection may lead to evolution towards novel adaptive peaks, which may lead to changes in former genetic constraints over time change, altering the line of least resistance (Figure 1c & 2). Alternatively, former phenotype-genotype covariations might become relaxed, leading to a higher potential for phenotypic diversification (Bacigalupe, 2009).

Despite our understanding of the different stages of adaptive radiations, it is much less clear which extrinsic factors may actually trigger the evolution and the extent of ecological diversification and finally initiates an adaptive radiation (Losos, 2010; Nosil, 2012). Several major features driving a radiation have so far been identified, including niche dimensionality and the strength of the ecological gradient that can shape the extent of diversification (Nosil & Sandoval, 2008; Gavrilets & Losos, 2009). Theoretical models furthermore implicate that the

extent of adaptive diversification, i.e. the number of emerging ecotypes, increases with both the number of potential niches and habitat size, which both approximate ecosystem size (Gavrilets & Vose, 2005). In addition, sexual selection may promote diversification by reducing gene flow between ecotypes (Maan & Seehausen, 2011). Indeed, all these factors have been found to explain the presence and the extent of adaptive radiations in African cichlid fishes (Wagner *et al.*, 2012). Adaptive radiation may be furthermore initiated by early ecological release, e.g. through the loss of competition upon colonizing a novel environment (Mayr, 1963; Bolnick *et al.*, 2010).

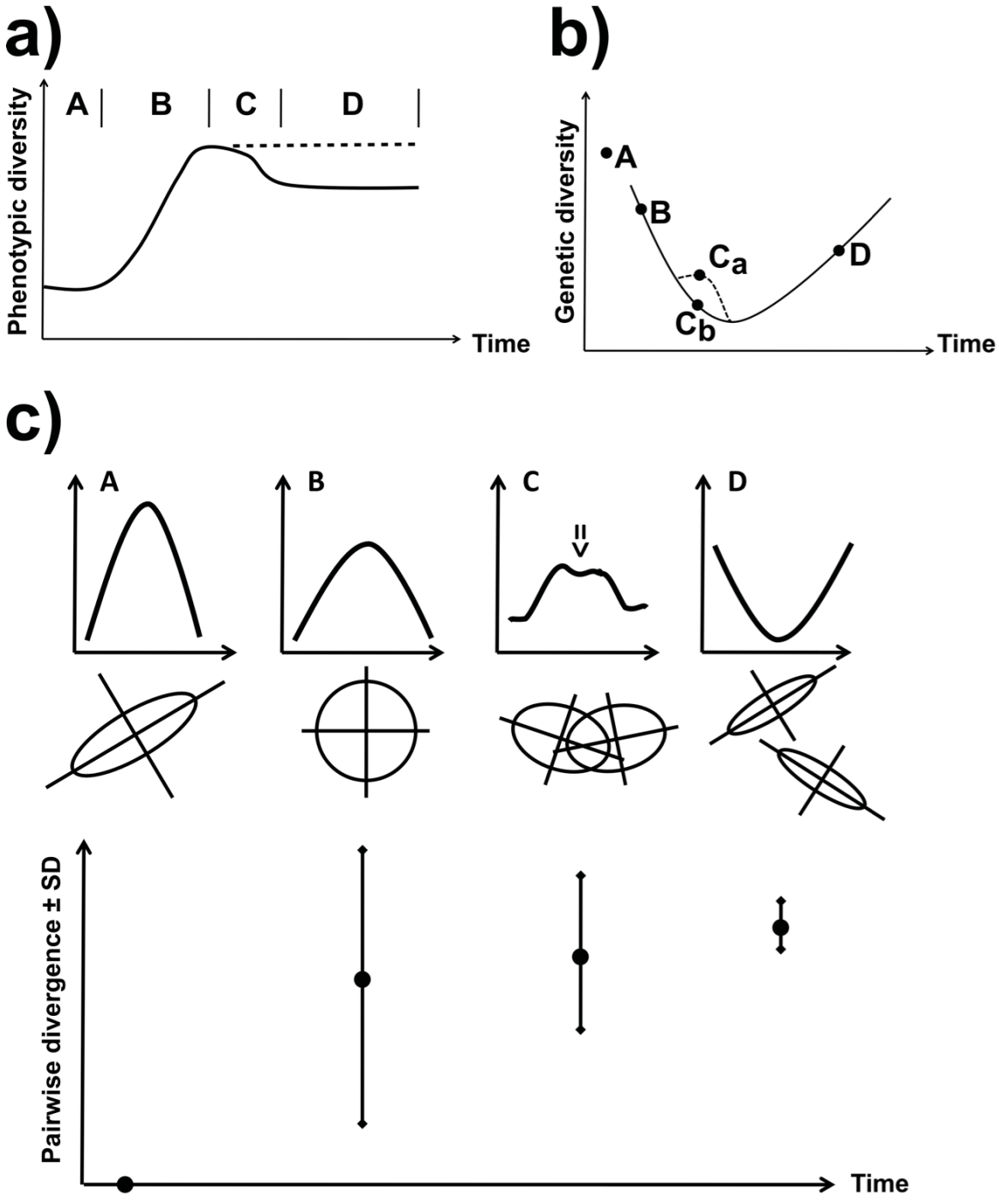

Figure 1: *Phenology of phenotypic and genetic variation and divergence during an adaptive radiation over time. Time axes are not equal among graphs and depend on the processes involved, i.e. the relative amount of phenotypic plasticity or standing genetic variation (see text for details).*

a) Relative standing phenotypic variation within an adaptive radiation over time. ***A*** *early invasion stage with a lag phase, where the population undergoes a bottleneck event, which decreases the amount of adaptive standing phenotypic variation.* ***B*** *populations experiencing a relaxed selection regime and potentially a release of former constraints adapt towards novel parts of the available morphospace. Rapid niche occupation and speciation occurs.* ***C*** *species may overshoot the sustainable maximum, leading consequently to the extinction of some species. The amount of phenotypic diversity may decrease (solid line) or reaches an equilibrium state (dashed line).* ***D*** *late stage of an adaptive radiation, where balancing selection keeps the total morphospace usage in a stasis, potentially with a slight increase in standing variation over time through neutral phenotypic evolution.*

b) Genetic diversity during the early stages of an adaptive radiation followed by a biological invasion event: ***A*** *ancestral population with large standing genetic diversity.* ***B*** *introduced population with a subsample of the ancestral gene pool. This population either experiences a lag phase where standing genetic diversity is acquired through mutation or eventually hybridization, followed by a range expansion* $\boldsymbol{C_a}$ *or the genetic bottleneck may remove prior constraints, followed by a range expansion* $\boldsymbol{C_b}$*. Over time, genetic diversity may increase through mutation or hybridization* ***D****.*

c) Changes of selection regimes and concomitant phenotypic differentiation towards two adaptive peaks. Panels on top depict hypothetical frequency distributions of adaptive traits with their associated variance/covariance matrix, indicating the potential for phenotypic evolution. The lower panel shows phenotypic divergence for two evolved ecotypes (note that at the beginning, only one ecotype is present) with the corresponding standard deviation. ***A*** *stabilizing selection in the ancestral range of a species, which occupies only a single niche. After the colonization of a new habitat the species experiences first relaxed selection regime* ***B****, which leads to an increase in the extent of the occupied space on the adaptive landscape. Populations sampled in two distinct habitats are likely to be divergent but the phenotypic distributions are likely to overlap to a large extent. Natural selection within the newly occupied niches favors different phenotypes, and hence decreases the phenotypic overlap, leading to a bimodal trait distribution (****C*** *and* ***D****).*

Biological invasion

Biological invasions can be defined as the introduction and the subsequent spread and expansion of a species outside of its native range (Prentis *et al.*, 2008). Many invasions are characterized by the emergence of rapid phenotypic changes that are associated with adaptation to ecologically contrasting environments, especially between the invaded and native range

(Elton, 1958; Herrel *et al.*, 2008; Keller & Taylor, 2008; Calsbeek *et al.*, 2011). Similar adaptations may furthermore occur within the invaded range itself (Hendry *et al.*, 2000; Phillips *et al.*, 2006). Although the importance of such evolutionary changes within the invaded range itself have been already recognized in the 1970ies (Baker, 1974), they have become only extensively studied over the last two decades (Sakai *et al.*, 2001; Carroll *et al.*, 2007; Davidson *et al.*, 2011; Westley, 2011). The evolutionary processes that trigger a successful invasion can be characterized as different stages, ranging from a species' introduction to its successful range expansion and subsequent adaptation to novel environments (Theoharides & Dukes, 2007; Prentis *et al.*, 2008). The successful progression along these stages depends on many intrinsic and extrinsic factors, which may each impede a species from becoming invasive. Indeed, only a very limited fraction of introduced species becomes actually invasive (Lockwood *et al.*, 2007). The evolutionary stages and processes that underlie a successful invasion are furthermore similar to the early stages of adaptive radiations (Yoder *et al.*, 2010; Losos, 2010; Figure 1) and contemporary ecotype formation (see Hendry *et al.*, 2000; Koskinen *et al.*, 2002 as examples and Carroll *et al.*, 2007; Westley, 2011 for a review). The process of ecological speciation involves analogous steps as for biological invasions that may either promote or impede progression (Nosil *et al.*, 2009). Hence, biological invasions provide an opportunity to study the evolutionary mechanisms that may generally underlie contemporary adaptive divergence in wild populations and the emergence of new species.

The initial colonization success of an introduced species may strongly depend on its adaptive genetic potential, which allows it to adapt to a novel environment (Lee, 2002; Barrett & Schluter, 2008). Indeed, propagule pressure, i.e. the initial population size, is a major factor that determines the adaptive potential and success of an introduced species, where selection can rapidly act on preexisting adaptive alleles that were already screened by selection in the past (Sakai *et al.*, 2001; Simberloff, 2009). However in many cases, the founder populations of biological invasions are often relatively small and gene flow from its ancestral population is limited or absent, leading to severe genetic bottlenecks (Lee, 2002; Prentis *et al.*, 2008; Dlugosch & Parker, 2008). Subsequent founder effects and inbreeding depression may further limit the ability of the introduced population to establish and expand. In many cases, introduced populations persist for some time only locally with a relatively small population size before they undergo a range expansion. This invasion stage has been commonly referred to as the "lag phase" (e.g. Sakai *et al.*, 2001; Prentis *et al.*, 2008) and may reflect the time that is required for adaptive evolution to overcome potential genetic constraints (Sakai *et al.*, 2001; Theoharides & Dukes, 2007).

Adaptive genetic variation may however be rapidly increased through intra- and interspecific admixture and hybridization, alleviating the initial effects of bottlenecks (Reznick & Ghalambor, 2001; Lee, 2002; Prentis *et al.*, 2008). The introduction of multiple populations from different parts of the native range can similarly increase the standing genetic variation in the introduced range, which may even exceed the variation observed in any of the ancestral populations

(Kolbe *et al.*, 2004; Lavergne & Molofsky, 2007). Hybridization may furthermore increase the evolutionary potential of an introduced species, because hybrids may express novel trait combinations that are outside the phenotypic distribution of either parental species through transgressive segregation and that potentially allow them to colonize novel habitats and niches (Vellend *et al.*, 2007; Stelkens & Seehausen, 2009a). Classical examples for invasive species of hybrid origins derive from cordgrass (*Spartina sp.*; Ainouche *et al.*, 2009), sunflowers (*Helianthus* sp.; Rieseberg *et al.*, 2003) or sculpins (*Cottus* sp.; Nolte *et al.*, 2005). In these cases, hybrids of different parental species form distinct phenotypes that have invaded novel environments outside of the parental range. Alternatively, rapid adaptation to a new environment can be realized through adaptive phenotypic plasticity (West-Eberhard, 2003; Richards *et al.*, 2006; Ghalambor *et al.*, 2007; Davidson *et al.*, 2011; Westley, 2011). In this case, adapted phenotypes that match the novel environment the best can rapidly emerge, leading to a fast range expansion as well as the potential for rapid subsequent adaptation to distinct parts of the invaded range. Indeed, plasticity seems to be a main driver in many biological invasions (Davidson *et al.*, 2011). Over time, such adaptive plastic responses may then become genetically determined through canalization, depending on the stability of the selective regime and the costs for plasticity (Lande, 2009). Plasticity can however only promote rapid range expansion if it places a population close enough to a new phenotypic optimum (Ghalambor *et al.*, 2007). The rapid adaptive plastic response in such cases is likely a product of past selection events and hence analogous to the "flexible-stem" theory, which predicts that adaptive radiations can be initially seeded by a species that has much ancestral plasticity retained (West-Eberhard, 2003; Ghalambor *et al.*, 2007; Wund *et al.*, 2008, see below). Alternatively, plasticity may need to evolve itself, which theoretically occurs over relatively short time scales (Lande, 2009; Thibert-Plante & Hendry, 2011).

Once the introduced species has overcome the aforementioned constraints, it can undergo a range expansion, rapidly colonizing the available and suitable habitat. The colonization of heterogeneous environments may further cause disruptive and diversifying selection, causing diversification within the invaded range (Vellend *et al.*, 2007). Expansion and subsequent adaptation to several distinct niches may in addition lead to directional selection between subpopulations, being divergent between niches, as they reach carrying capacity. This can furthermore result in changes of the genetic variance-covariance structure, i.e. the underlying evolutionary constraints (Bacigalupe, 2009, analogous to Figure 1c. Such divergent selection between niches may then lead to the formation of distinct ecotypes and ultimately ecologically differentiated species (Simpson, 1953; Schluter, 2000; Yoder *et al.*, 2010). The potential for local adaptation and diversification may however be decreased close to the boundary of the invaded range if the adaptive genetic potential becomes reduced during expansion (Garcia-Ramos & Rodriguez, 2002). On the other hand, continuous gene flow from the central population may similarly impede local adaptation (Kirkpatrick & Barton, 1997).

In addition to these intrinsic factors, environmental dependent extrinsic factors may similarly impede or promote the success and progression of an

introduced species. In many cases, an introduced population may initially experience ecological release with a relaxed selection regime due to the absence of former competition from other species, predation or parasites (Blossey & Nötzold, 1995; Lahti *et al.*, 2009). In this context, the evolution of increased competitive ability (EICA) hypothesis has been postulated, which predicts that the loss of enemies causes the introduced species to evolve a reallocation of resources from former necessary defense mechanisms to greater competitive ability, assuming a tradeoff between these two traits (Blossey & Nötzold, 1995). Ecological release may further promote niche expansion and the colonization of additional habitats (Sakai *et al.*, 2001; Bolnick *et al.*, 2010). Changes in the local environment and interspecific interaction during range expansion may on the other hand hamper a species from spreading further (Theoharides & Dukes, 2007). For example, native species may coevolve, leading to increased competition and/or predation (Vellend *et al.*, 2007).

A successful invasion and potentially diversification of a species can affect the evolutionary fate of the coexisting native species in several additional ways. For example, invasive species may have severe impacts on the ecosystems that they invade, by changing the adaptive landscape and the previously established food web structure (Vellend *et al.*, 2007; Simberloff, 2011). This has been shown for the invasive and now widely distributed zebra mussel (*Dreissena polymorpha*), which is a selective filter feeder on smaller planktonic prey. The presence of zebra mussels can consequently change the size and species distribution of the plankton community that is available to other species (Strayer, 2009; Hirsch *et al.*, 2013). By forming large colonies, zebra mussels provide furthermore additional niche space for small benthic invertebrates, increasing their abundance and diversity (Strayer, 2009). The combination of these effects can lead to an altered prey community for top consumers, inducing divergent selection pressures (Hirsch *et al.*, 2013). Invaded or introduced species may on the other hand provide themselves novel niches for native species and in such cases promote evolutionary responses of native species (Vellend *et al.*, 2007). Empirical evidence comes from the recent host shift of the apple maggot fly *Rhagoletis pomonella* from North America that feeds naturally on hawthorn, but which evolved genetically distinct ecotypes that are specialized to feed on the introduced apple (Feder *et al.*, 1988; Michel *et al.*, 2010). Another example is the North American soapberry bug (*Jadera haematoloma*), which evolved different beak lengths to feed on fruits from different introduced tree species (Carroll *et al.*, 2001).

Mechanisms

Standing genetic variation

The successful colonization of novel environments and the subsequent adaptation to them can be either realized through new mutations or by selection on preexisting standing genetic variation (Barrett & Schluter, 2008). Depending on the underlying mechanism, the time for an adaptive response and successful colonization differs: On the one hand novel and beneficial mutations may require

a relatively long waiting time to arise. Consequently genetically depauperate populations would need time for advantageous genetic variation to arise, a process thought to be partly responsible for the lag phase during biological invasions (Sakai *et al.*, 2001). On the other hand, a species may undergo a range expansion relatively fast if standing genetic variation in adaptive genes permits the emergence of beneficial phenotypes and their exposure to selection (Facon *et al.*, 2006; Barrett & Schluter, 2008). Adaptation from standing genetic variation can be furthermore fast because beneficial alleles occur initially at a higher frequency than the ones, which emerged through mutation. This can then lead to the rapid emergence of different phenotypes that are adapted to distinct habitats within the colonized area. In addition, sorting of such preexisting alleles can in principle rapidly lead to adaptive and heritable phenotypic differentiation between these populations (Nosil, 2012; see Barrett *et al.* 2008 for an example).

The level of standing genetic variation can be additionally increased through gene flow between distinct populations as well as through intra- or interspecific hybridization (Seehausen, 2004; Barrett & Schluter, 2008; Nolte & Tautz, 2009; Schluter & Conte, 2009). This may further increase the adaptive potential of the admixed population because most of the genetic variation has been previously screened by selection and may thus fuel the colonization and subsequent diversification of a novel environment. Indeed, admixture and hybridization can lead to the emergence of distinct species (Rieseberg *et al.*, 2003; Nolte *et al.*, 2005) and may even seed entire adaptive radiations (Seehausen, 2004).

Phenotypic plasticity

The role of phenotypic plasticity, i.e. the ability of a single genotype to form distinct phenotypes in either promoting or constraining adaptive evolution has been a long debate (Baldwin, 1896; Scheiner & Lyman, 1989; West-Eberhard, 2003; Pigliucci, 2005; Lande, 2009; Thibert-Plante & Hendry, 2011). On the one hand, plasticity can lead to a fast expression of beneficial phenotypes, where a single genotype would be able to express different phenotypes, each of which is favorable in a different environment. Such divergent trait expression between habitats can itself become genetically fixed over time through phenotypic canalization, depending on the strength of selection and the costs of maintaining plasticity (Yeh & Price, 2004; Lande, 2009). Once emerged, divergent selection on the distinct phenotypes may lead to the emergence of prezygotic reproductive isolation (Thibert-Plante & Hendry, 2011). The effect of plasticity on the strength of divergent selection depends however on the timing at which plasticity occurs, i.e. if plasticity is expressed early in ontogeny before possible dispersal between contrasting habitats, divergent selection can be strong because selection against immigrants can occur, whereas expression after dispersal may dissipate divergent selection (Thibert-Plante & Hendry, 2011). On the other hand plasticity may hamper adaptive diversification because it can shield the genome from the effects of selection (Ghalambor *et al.*, 2007; Thibert-Plante & Hendry, 2011).

Empirical evidence suggests that in many cases plasticity is adaptive (Day *et al.*, 1994; Robinson & Wilson, 1996; Aubret *et al.*, 2004; Lundsgaard-Hansen *et al.*, 2013) and can promote the colonization of novel environments (Yeh & Price, 2004; Richards *et al.*, 2006). Because plasticity can act rapidly, it has been especially invoked to play a major role in evolutionary young systems, such as invasive species (Richards *et al.*, 2006; Davidson *et al.*, 2011) or during the formation of distinct ecotypes of fishes during the postglacial colonization of freshwater bodies (Smith & Skúlason, 1996; Wund *et al.*, 2008). Indeed, invasive species seem to have a significantly higher level of plasticity than their native counterparts (Davidson *et al.*, 2011). Similarly, following the "flexible-stem" theory, adaptive radiations can be initially promoted by phenotypic plasticity, where a radiation is seeded by a species that has much ancestral plasticity retained (West-Eberhard, 2003). The successful expansion and formation of ecologically distinct adaptive phenotypes is then facilitated. Empirical evidence for the flexible-stem theory and the importance of plasticity in initiating adaptive radiations derives especially from evolutionary young systems, such as the postglacial colonization of freshwater habitats by fishes (Smith & Skúlason, 1996; Wund *et al.*, 2008) or life history-changes in translocated snakes (Aubret *et al.*, 2004).

Quantitative genetics

The higher dimensionality of multivariate selection can be visualized using the metaphor of an adaptive landscape or fitness landscape that is usually projected into a two- or three-dimensional space (Wright, 1932; Steppan *et al.*, 2002; Gavrilets, 2004). Such landscapes consist of peaks with increased fitness that are separated from each other by valleys of lower fitness, resulting in a rugged pattern. Although theory suggests that alternative adaptive landscapes may exist that could for example be rather flat under neutral assumptions or "holey" (Gavrilets, 1997, but see Gavrilets, 2004 for a review), rugged landscapes have been widely used in a theoretical (Lande, 1979; Steppan *et al.*, 2002) and empirical (e.g. Schluter, 1996; Eroukhmanoff & Svensson, 2011, Chapter 2 & 6) context. Adaptive evolution within a given landscape can be interpreted as climbing up such adaptive hills (*sensu* Gavrilets, 2004), where selection may prevent a further movement once a local peak is reached. A population within an adaptive landscape can be quantified by its G matrix, which is based on the additive genetic variance/covariance of traits. Quantitative genetic theory here predicts that the evolution towards adaptive peaks is thought to progress along so called "lines of least resistances" or g_{max}, which can be quantified as the leading eigenvector of the G matrix (Lande, 1979; Schluter, 1996, see Steppan *et al.*, 2002 for a review). Biologically, the leading axis g_{max} comprises most of the genetic variation, shaped by selection and drift, and reflects the underlying genetic constraints within a population and represents the major axis of genetic constraints (Marroig & Cheverud, 2005). The directionality of G matrices as well as the evolution of G over time can consequently be compared by calculating the angle θ between different g_{max} (Schluter, 1996, but see Roff *et al.*, 2012 for a methodological overview).

Because pleiotropic effects may constrain evolution away from g_{max}, short-term evolution is predicted to follow the direction of this leading axis. Recent empirical evidence further suggests that G matrices may change rapidly, especially during the colonization of novel environments (Bacigalupe, 2009; Calsbeek *et al.*, 2011; Eroukhmanoff & Svensson, 2011, Chapter 2 & 5) as well as within the same environment (Bjorklund *et al.*, 2013). Through time, selection may commonly change the direction of g_{max} towards an existing or a new optimum on the adaptive landscape. Neutral processes, such as drift and mutation, can similarly change the G matrix and hence g_{max} over time (Chapuis *et al.*, 2008).

In the absence of quantitative genetic data, the G matrix can be approximated by the P matrix, which is based on phenotypic data from wild populations (Arnold *et al.*, 2008). Here, P is defined as the combination of the genetic and environmental covariance matrices, i.e. G + E (Lande, 1979; Arnold & Phillips, 1999), where both may furthermore interact (G x E; Falconer, 1989). Consequently, P matrices potentially include phenotypically plastic traits, that are differentially expressed in divergent environments (Pigliucci *et al.*, 1999). The leading eigenvector of a P matrix (p_{max}) may therefore serve as an overall measurement of phenotypic variation observed in the wild, accounting for both genetic and environmental constraints. However, evolutionary patterns of p_{max} differ between plastic and genetically determined traits, which becomes apparent when studying parallel cases of phenotypic divergence along a temporal gradient. On the one hand, adaptive phenotypic plasticity can promote rapid phenotypic differentiation between distinct environments (Ghalambor *et al.*, 2007; Svanbäck & Schluter, 2012). Depending on the selective regime, genetic assimilation may reduce plasticity over time and replacing it by genetic evolution, while the phenotypes that were initially produced by plasticity are retained (Lande, 2009). Consequently p_{max} of different replicated systems that vary in their age should align, i.e. show a small or zero angle θ between them, if each system experiences a comparable selective regime (Figure 2b). However, because phenotypic plasticity can evolve itself as an adaption to a novel environment, initial differentiation of p_{max} between an ancestral and a newly adapted phenotype may occur (Lande, 2009; Svanbäck & Schluter, 2012; Draghi & Whitlock, 2012). In such a case, the angle θ between the ancestral and its derived p_{max} should initially increase from generation to generation, mimicking a genetically determined scenario over the first dozens of generations as described below (Lande, 2009). Once evolved, subsequent p_{max} are expected to align.

On the other hand, assuming a comparable adaptive landscape and selective regime among replicates, θ between mainly genetically determined p_{max} should evolve over time through selection and drift (Schluter, 1996). Here, θ is expected to subsequently increase over time between an ancestral p_{max} and the p_{max} of a derived population that is evolving towards a new adaptive optimum (Schluter, 1996; 2000, Figure 2a). This has been shown in a comparative study among several vertebrate taxa, based on genetic distances to establish divergence time (Schluter, 1996). However, genetic distance may be affected by divergent selection and may therefore lead to a biased interpretation. Similarly, founder effects can induce rapid evolutionary changes during the colonization of

new environments, directing and changing the evolution of the underlying G matrix (Bacigalupe, 2009). In such cases, the genetic differentiation from an ancestral population remains low, despite a significantly diverged G matrix (Calsbeek *et al.*, 2011; Eroukhmanoff & Svensson, 2011). These potential caveats can however be overcome if similarly diverged systems of different geological age are studied.

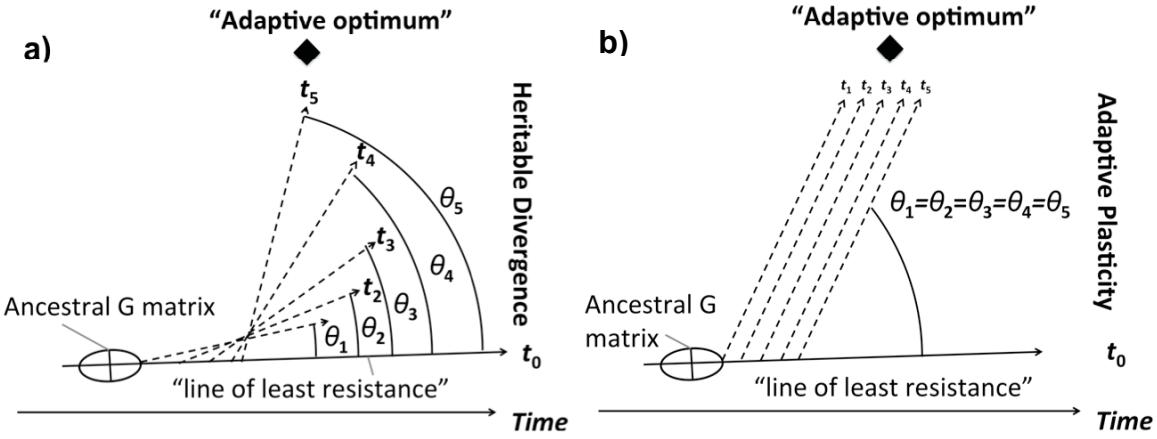

Figure 2: *The evolution of the P matrix and its underlying G matrix over time assuming that the underlying traits are either mainly genetically determined (a) or underlie adaptive plasticity. Shown is the ancestral G matrix with its line of least resistance or g_{max} (solid arrow) and the g_{max} of diverged populations sampled along a temporal gradient (t_1-t_5, dashed arrows) that evolve towards a novel adaptive optimum. The angle θ indicates the degree of divergence over time. Assuming mainly heritable determined traits θ is expected to evolve gradually over time, whereas θ aligns independently of time if the underlying traits are mainly plastic (see main text).*

The stickleback model system

The threespine stickleback (*Gasterosteus aculeatus* species complex) is an originally marine fish species with a circumpolar distribution in the Northern Hemisphere. Throughout its distribution, marine stickleback colonized freshwater habitats forming repeatedly an anadromous ecotype that spawns within freshwater (Bertin, 1925; Wootton, 1976; Jones *et al.*, 2006). Further adaptation led to the repeated evolution of purely freshwater dwelling populations that adapted to different habitats, such as lakes and streams. Here habitat dependent divergent selection lead to the repeated emergence of parapatric lake-stream systems (Berner *et al.*, 2009; Hendry *et al.*, 2009; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b), Chapter 3 & 6). In contrast, only few cases of coexisting ecotypes within a lake have been documented for stickleback so far. These include benthic and limnetic feeding species in Canadian lakes (Schluter, 2000; Gow *et al.*, 2008) and substrate specific ecotypes in Arctic lakes (Kristjánsson *et al.*, 2002a). In the latter case, sticklebacks have been shown to occupy different substrates and depth habitats in Icelandic lakes, being morphologically distinct from each other in their antipredator phenotypes as

well as in their feeding habits (Kristjánsson *et al.*, 2002a). Phenotypic divergence here is partially driven by different predator avoidance strategies against Arctic charrs (Doucette *et al.*, 2004). At least three substrate specific stickleback ecotypes have been described in Arctic lakes: a lava type, a mud type and a deep water dwelling type that forages in *Nitella* sp. meadows on mud substrate (Ólafsdóttir *et al.*, 2007a). The potential for prezygotic isolation between ecotypes has been furthermore indicated (Ólafsdóttir *et al.*, 2006).

Most of these systems evolved after the last glacial maximum 8'000-12'000 years ago (Schluter, 1993; Bell & Foster, 1994; McKinnon & Rundle, 2002; Kristjánsson *et al.*, 2002a). But several cases of contemporary evolution in stickleback have similarly been demonstrated, which mainly focus on the rapid emergence of freshwater-adapted populations from ancestral marine or anadromous colonizers, particularly in regard to phenotypes related to antipredator defense (Bell *et al.*, 2004; Gelmond *et al.*, 2009; Le Rouzic *et al.*, 2011). The rapid emergence of adapted phenotypes within freshwater has been related to phenotypic plasticity (Wund *et al.*, 2008; 2012) as well as the use of standing genetic variation within the marine population (Colosimo *et al.*, 2005; Jones *et al.*, 2012b). In the latter case, occasional genetic introgression between locally adapted freshwater populations and marine ancestral populations let to the persistence of alleles beneficial for freshwater adaptation in the ancestral marine gene pool (Jones *et al.*, 2012b). These can become selected again when new freshwater bodies are colonized, leading to the occurrence of similar and parallel evolved systems (Schluter & Conte, 2009). The replicated nature of these evolutionary diversification as well as the number of distinct, evolved ecotypes make the stickleback a model system in ecology and evolution (McKinnon & Rundle, 2002).

Another commonly observed axis of divergence, besides the marine-freshwater transition is the parapatric differentiation between lake dwelling populations and their associated streams (Hagen & Gilbertson, 1972; Gross & Anderson, 1984; McPhail, 1994; Berner *et al.*, 2008; Kaeuffer *et al.*, 2012; Ravinet et al., 2013b). Stream populations in these systems often exhibit morphological features that allows them to exploit benthic prey, while lake populations fall along a continuum between two possible ecotypes, one associated with the littoral zone and the other associated with the limnetic zone of lakes. Habitat dependent feeding related divergence may occur, where stream fish feed predominantly on benthic prey, whereas lake fish may specialize on a limnetic diet (Gross & Anderson, 1984; Berner *et al.*, 2008), but specialization can differ among seasons (Chapter 4). Several studies have demonstrated that such ecotypic divergence can emerge despite the high potential for gene flow in stickleback (Schluter & McPhail, 1992; Rundle *et al.*, 2000; Berner *et al.*, 2009). Habitat dependent parapatric divergent selection between lake and stream drives furthermore phenotypic differentiation in body shape due to different flow regimes (Reid & Peichel, 2010) and antipredator traits. The latter is driven by different predator communities among habitats, which exert divergent selection between them. Where lake dwelling populations are mainly exposed to a marine-like piscivorous predation regime of birds and fish, stream dwelling populations on the other hand experience a mainly invertebrate dominated predation community (Reimchen, 1994). Selection is consequently expected to

evolve decreased spine lengths in streams, reducing the potential for invertebrates to attach (Reimchen, 1980; 1994; Marchinko, 2009).

In Switzerland, the threespine stickleback had a naturally limited distribution, being reported until the 1870s only in the Rhine tributaries around Basel north of the Jurassic Mountains (Fatio, 1882), where the steepness of the Rhine probably prevented further spread (Lucek *et al.*, 2010). At that time, however, sticklebacks were released almost simultaneously in the upper Rhine River (Heller, 1870) upstream of Lake Constance in Austria, and in a stream near Geneva, connected to Lake Geneva in 1872 (Fatio, 1882). Additional releases in the Lake Neuchatel catchment and in the upper Rhone, upstream of Lake Geneva in the Valais, took place at the beginning of the 20th century (Bertin, 1925). During the first half of the 20th century, several aquarium fish traders used the Basel population as source population for distribution to aquarists (Steinmann, 1936). What followed was a rapid successful invasion of the entire Swiss midlands within the Rhine/Aare and the Rhone drainages, where stickleback became one of the most abundant fish species especially during the eutrophication period in the 1960ies and 70ies (Laurent, 1972; Numann, 1972). They now occupy a very wide range of different habitats including streams, ponds and the shores as well as the pelagic zone of large lakes (Lucek *et al.*, 2010, Chapter 2 & 3).

A former study has shown that the different introduction events within Switzerland that were described in historical literature derive from distinct European lineages as inferred by mitochondrial DNA (Lucek *et al.*, 2010). It was found that the Lake Geneva system was seeded by a lineage from the Southern Rhone, draining into the Mediterranean Sea, whereas individuals from Eastern Europe, belonging to the Baltic drainage were introduced in the Lake Constance region (Mäkinen & Merilä, 2008; Lucek *et al.*, 2010). Consistent with natural populations from their inferred place of origin, both lineages show distinct antipredator related lateral plate phenotypes being linked to distinct *Eda* alleles (Berner *et al.*, 2010). Here the individuals from the Constance region show a marine like fully plated freshwater phenotype, whereas individuals in the Geneva region are mainly low plated (but see Chapter 2), which is the common south European plate phenotype (Münzing, 1959). The third genetic lineage found in Switzerland occurs predominantly within the Basel region and is fixed for the low plated phenotype that was historically described in this region (Fatio, 1882). Therefore it likely originates from a natural colonization along the Rhine River from the North Sea. All mitochondrial haplotypes furthermore coexist in the central part of Switzerland, where in addition nuclear genetic marker indicate admixture between the distinct lineages (Lucek *et al.*, 2010; Berner *et al.*, 2010, Lucek *unpublished data*). Throughout the admixture zone, an increase in phenotypic variation is observed for lateral plate phenotypes, where in addition to the low and highly plated phenotypes, intermediate forms are present (Lucek *et al.*, 2010). The increase in adaptive standing genetic variation may have further fuelled habitat dependent divergence by increasing the adaptive potential to face distinct selective pressures. Habitat dependent selection on antipredator traits has indeed been suggested in several systems (Lucek *et al.*, 2010; Zeller et al., 2012a, Chapter 2) but its underlying selective regime is not yet understood (Zeller et al., 2012b).

The physical contrast between some of the divergent habitats where stickleback established resembles those typically associated with ecological habitat dependent divergence in the natural range of the species (McKinnon & Rundle, 2002; Hendry *et al.*, 2009; Berner *et al.*, 2009; Kaeuffer *et al.*, 2012; Ravinet et al., 2013b). Especially phenotypic divergence between parapatric lake and stream populations can be repeatedly observed (Berner *et al.*, 2010, Chapter 2-5, Figure 3). Parapatric divergence lead furthermore to the evolution of distinct life history strategies (Moser *et al.*, 2012, Lucek *unpublished data*) and may be adaptive (Chapter 4). Given the short evolutionary time, i.e. less than 140 years, since the emergence of these ecologically diverged stickleback populations, Swiss stickleback provide a great opportunity to study the evolutionary processes that underlie rapid ecotype formation during a biological invasion, which can be compared to analogous systems from across the natural range that differ in their respective evolutionary age.

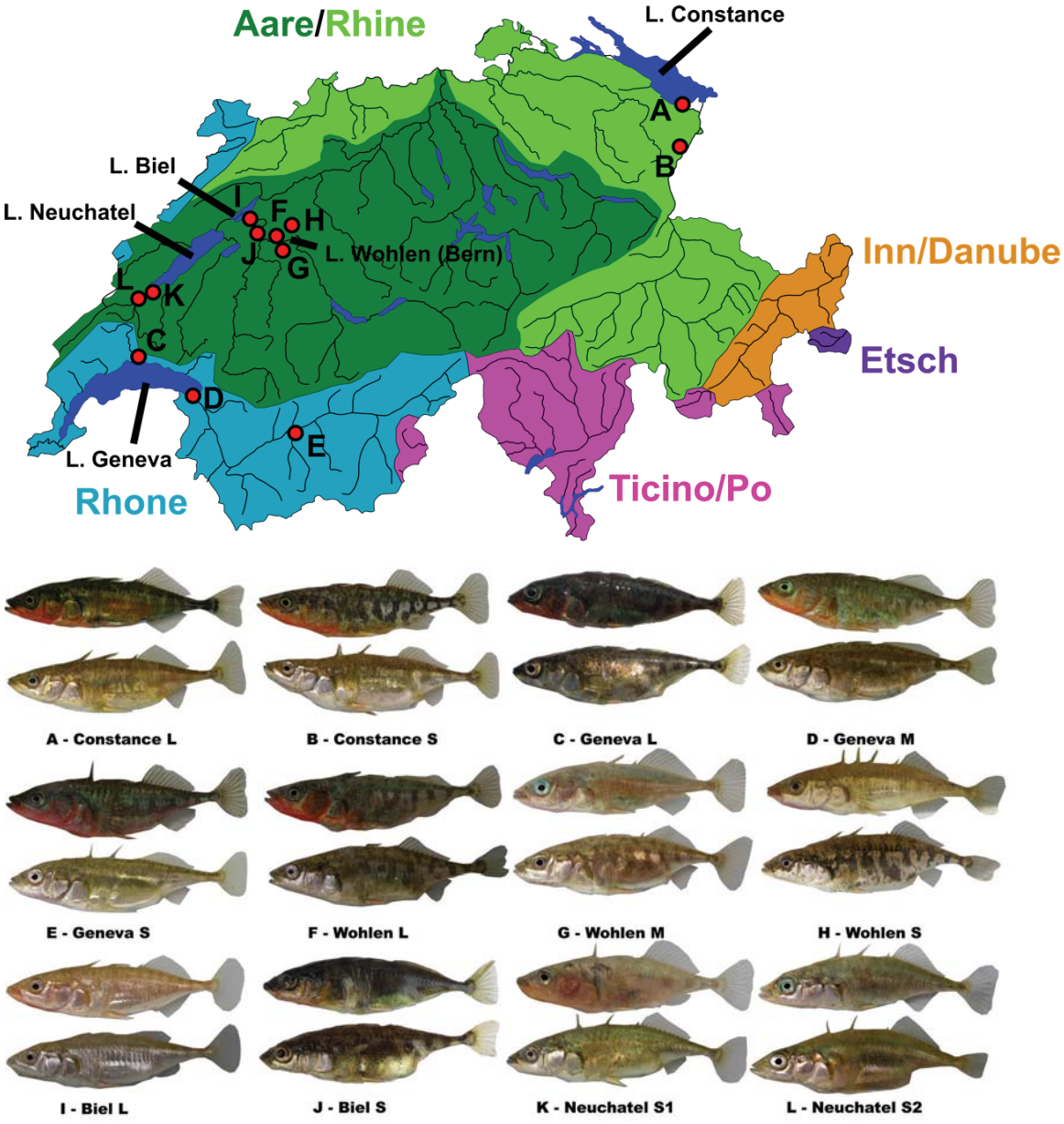

Figure 3: *Top: Map of Switzerland with the major watersheds being indicated in different colors: A – Constance L; B – Constance S; C – Geneva L; D – Geneva M; E – Geneva S; F – Bern L; G – Bern M; H – Bern S; I – Biel L; J – Biel S; K – Neuchatel S1 (near lake); L – Neuchatel S2 (upstream). Sample sites belong to the Rhine drainage (A, B); Rhone drainage (C-E), Aare drainage (F-J), or the Orbe drainage (K, L) respectively (© Wikimedia). Bottom: Representative examples of stickleback from different Swiss populations. Shown are each a male (top) and a female (bottom) for twelve populations that either derive from a lake (L) or a stream (S) site. Populations are consistent with the ones used in Chapter 1. Individuals are not scaled to size.*

Thesis outline and summary of the chapters

Invasion

Chapter 2 focuses on the Lake Geneva system, where habitat dependent phenotypic and ecological divergence occurs between contemporary lake and stream dwelling stickleback populations. Using phenotypic data collected from historical museum collections, we aim to reconstruct the evolution of habitat dependent phenotypic divergence through time. Specifically we ask if habitat dependent divergence occurred already shortly after sticklebacks were introduced or if the divergence evolved over time. Using genetic data of contemporary populations, we furthermore infer the underlying invasion history. Based on quantitative genetic approaches, we indeed find that parapatric divergence between habitats can occur consistently and independently of time, but that the patterns depend on the trait combinations studied. Here the evolutionary line of least resistance differs especially for antipredator related phenotypic traits consistently between lake and stream habitats independently of their age, suggesting that habitat dependent selective regimes reorient the underlying G matrix rapidly towards distinct adaptive peaks. This phenotypic divergence may have been fuelled by secondary gene flow from outside the Lake Geneva region, which introduced new alleles of major phenotypic effects at the *Eda* locus. Whereas historically only the low plated phenotype (see above for details) has been described within the Lake Geneva region, lake fish show a significant increase in lateral plates especially among contemporary populations, which is concomitant with the presence of the respective *Eda* allele. Consequently our results suggest that divergent selection between habitats can quickly lead to some phenotypic differentiation among populations inhabiting these habitats, but that an increase in genetic variation through hybridisation can promote further divergence.

The potential for contemporary ecological divergence between parapatric lake and stream habitats is revisited in **Chapter 3** but the focus is expanded to the overall previously described Swiss stickleback invasion (Lucek *et al.*, 2010). Specifically we test if the colonization of distinct habitats is generally associated with a parapatric phenotypic contrast and to which extent this contrast occurs in parallel among lake-stream systems. Using linear and shape morphology as well as stable isotopic data we compare the degree of parapatric divergence in relation to neutral genetic divergence. By comparing the degree of phenotypic

and genetic divergence in relation to environmental gradients among parapatric systems we further infer to which extent ecological divergence can be predicted and if ecological adaptation lead to a reduction of gene flow among parapatric populations through isolation by adaptation (Nosil *et al.*, 2008; Shafer & Wolf, 2013). We find consistent phenotypic divergence between populations occupying distinct habitats. This involves parallel evolution in several traits with known ecological relevance in independent evolutionary lineages. Importantly, habitat dependent divergence occurs consistently with the results in Chapter 2, mainly in antipredator related traits, which indicates a comparable selective regime among lake-stream habitats. Adaptive divergence supersedes homogenizing gene flow even at a small spatial scale. We find evidence that adaptive phenotypic divergence places constraints on gene flow over and above that imposed by geographic distance, signaling the early onset of ecological speciation.

Adaptation

In **Chapter 4** we test if the observed habitat dependent parapatric differentiation (see Chapter 3) is actually adaptive and hence shows a heritable component rather than being the result of adaptive phenotypic plasticity. To differentiate either effect, we used a controlled laboratory experiment with full-sib crosses of our formerly studied lake and stream stickleback populations in the Lake Constance catchment that showed a high degree of phenotypic, ecological and genetic differentiation. We infer fitness and hence adaptivity by comparing the relative growth rates of each population fed on either a foreign or native food resource that we identified from stomach contents of wild type individuals. We find that in the lake like food treatment lake fish grow significantly faster than stream fish, which is congruent with the inferred growth patterns among wild type individuals based on otoliths. In the stream like food treatment on the other hand, individuals from both populations grow similarly. Together, these results suggest that genetically determined and hence adaptive diversification has indeed occurred within less than 140 years since stickleback were introduced in the Lake Constance system, where stickleback from distinct habitats evolved furthermore different life history strategies (Lucek et al., 2012a; Moser *et al.*, 2012).

Using the same experimental individuals as in the former chapter, we test in **Chapter 5** explicitly to which degree plasticity and genetic determination as well as their interaction contribute to the observed phenotypic habitat dependent divergence separately for each phenotypic trait. We furthermore relate the changes observed among our experimental groups with wild type phenotypes of different age classes. Doing so we find that our experimental groups show indeed similar phenotypic divergence as observed in the wild, which suggests that habitat dependent divergence in feeding related traits is a main driver of phenotypic divergence in this system. Disentangling plastic and genetically determined effects, we find that contemporary ecotype formation is characterized by a combination of both plasticity and heritable divergence. The relative contribution of each differs among the traits studied, with traits related to the biomechanics of feeding showing strong genetic predisposition, whereas

traits related to locomotion are mainly plastic. These patterns differ furthermore between linear morphology and shape related traits, where the latter show an increased propensity for genetic determination. Nevertheless, these results implicate the interplay of both phenotypic plasticity and standing genetic variation can promote rapid ecotype formation during biological invasions.

Diversification

Where Chapters 2-5 focused on the evolutionary aspects of rapid and contemporary parapatric ecotype formation, it is necessary to embed these results in a broader evolutionary context, i.e. along a temporal axis, in order to test if they hold up over an extended evolutionary time. In **Chapter 6** we compare three parapatric lake-stream systems from Switzerland with three systems from Iceland that vary in their evolutionary age, forming a temporal gradient. Based on quantitative genetic approaches we test if and to which extent parapatric divergence occurs in parallel and if the underlying evolutionary trajectories are comparable. Finally we investigate how the habitat dependent ecotype formation in freshwater habitats is related the ancestral marine population. In both cases, we expect to see either a gradual evolution of the underlying evolutionary trajectory if the adaptive traits are mainly heritable or an alignment of these trajectories, independent of time (see Figure 2). We find that strong and consistent phenotypic divergence occurred independent of our studied temporal gradient for both the parapatric lake-stream divergence as well as for the marine-freshwater transition. Parapatric divergence between lake and stream populations seems to furthermore proceed along common evolutionary trajectories for certain trait combinations, especially for feeding and defense related traits. However, the degree of parapatric divergence differs across the investigated trait combinations, suggesting different evolutionary pathways and constraints among freshwater systems despite common evolutionary trajectories. In contrast, the dimensionality of ecotypic divergence was highest in our oldest systems and only there parallel evolution of unrelated ecotypes was strong enough to overwrite phylogenetic contingency. Interestingly also, the dimensionality of divergence in different systems varies between trait complexes, suggesting different constraints and evolutionary pathways to their resolution among freshwater systems.

Where the former chapters have focused on parapatric diversification, the possibility of sympatric diversification within a lake has so far been neglected. Although cases of discrete intraspecific intralacustrine diversification have only rarely been documented for stickleback (Taylor & McPhail, 2000; Kristjánsson *et al.*, 2002a), ecologically different modes may exist within a lake due to individual specialization (Snowberg & Bolnick, 2008). These may form distinct phenotypic clusters, which marks a progression along the continuum along which ecological speciation unfolds itself (Hendry *et al.*, 2009; Nosil, 2012). In **Chapter 7** we are using a novel clustering method to estimate the number of distinct phenotypic modes within nine independent lake systems and one ancestral marine population in Iceland. Substrate specific phenotypic intralacustrine diversification had previously been suggested for some of these lakes. Using the inferred number of phenotypic modes, we then test for an association between

ecological opportunity, gene flow from an ancestral gene pool and phenotypic diversification. We find phenotypic intralacustrine diversification and differentiation from the ancestral marine population to be the rule rather than the exception with ecologically relevant phenotypic traits differing among phenotypic modes. Eco-phenotypic diversification has occurred in parallel in the different lakes, with indications of non-random mating, inferred from neutral genetic markers, in four out of nine studied lakes and indications of reproductive isolation between phenotypic modes in two. Although neither the phenotypic variation nor the number of phenotypic modes in lakes were associated with any of our environmental variables, the dimensionality of phenotypic differentiation between ecotypes was significantly positively related to ecosystem size, and reproductive isolation was only found in the largest lakes. Overall, the existence of distinct phenotypic clusters in the absence of strong genetic divergence between them, suggests furthermore a relatively early stage of diversification along the ecological speciation continuum.

Conclusions & Synthesis

Since the publication of the seminal book *The ecology of invasions by animals and plants* by Charles S. Elton (1958), the study of the evolutionary processes that underlie biological invasions has gained increased attention (e.g. (Baker, 1980; Carroll & Dingle, 1996; Sakai *et al.*, 2001; Yeh & Price, 2004; Prentis *et al.*, 2008; Bacigalupe, 2009; Calsbeek *et al.*, 2011; Westley, 2011; Gurevitch *et al.*, 2011). Many biological invasions have consequently become unintentional evolutionary experiments that allow to test specific evolutionary predictions in the wild and help to understand the evolutionary mechanisms underlying contemporary ecotype formation and rapid evolution (Prentis *et al.*, 2008; Westley, 2011). Only relatively recently however, attempts were made to integrate the theory of invasion biology into other, more evolutionary frameworks such as the theory of adaptive radiations and the emergence of rapid ecotype formation (Carroll *et al.*, 2007; Yoder *et al.*, 2010; Westley, 2011; Abbott *et al.*, 2013). In this thesis, I aimed to bridge the gap between the fields of biological invasions and evolutionary ecology in the context of contemporary ecotype formation at the very early stage of adaptive radiations integrating among others quantitative genetic methods.

Evidence for rapid adaptive evolution

Over the last decades it has become increasingly evident that adaptive phenotypic evolution can be fast, where a single species may evolve different ecotypes over only a few generations in response to habitat alterations or following its introduction into a novel environment (e.g. Thompson, 1998; Hendry *et al.*, 2000; Huey *et al.*, 2000; Palumbi, 2001; Koskinen *et al.*, 2002; Hairston *et al.*, 2005; Hendry *et al.*, 2008; see Hendry *et al.*, 2007; Carroll *et al.*, 2007 for a review). However, whereas phenotypic and evolutionary divergence of an introduced population away from its ancestral population has been demonstrated a number of times (Phillips *et al.*, 2006; Carroll *et al.*, 2007; Keller

& Taylor, 2008; Prentis *et al.*, 2008), contemporary ecotype formation between distinct habitats *within* an invaded range has far less been documented (see (Keller & Taylor, 2008) for a review). For example introduced sockeye salmon (*Oncorhynchus nerka*) from Lake Washington evolved reproductive isolation between divergent reproductive environments over only 13 generations (Hendry *et al.*, 2000). Similarly positive natural selection lead to adaptive population differentiation in introduced graylings (*Thymallus thymallus*) in Norway in less than 120 years (Koskinen *et al.*, 2002). For most known examples, the level of replication is however rather limited, i.e. evidence for contemporary evolution derives either from single occurrences (Hendry *et al.*, 2000; Koskinen *et al.*, 2002) or very few replicates (Eroukhmanoff *et al.*, 2009). This lessens the extent on which general conclusions can be drawn on the ubiquity and parallelism of such events. Likewise the evolutionary trajectories that underlie such rapid evolutionary responses have only very rarely been studies (Badyaev, 2010; Eroukhmanoff & Svensson, 2011; Bjorklund *et al.*, 2013).

Throughout this thesis, we see evidence for ecological driven phenotypic differentiation, which is potentially adaptive. However, do the observed patterns provide enough evidence to categorize our studied systems as the onset of an adaptive radiation following the criteria outlined in the introduction? Several lines of evidence seem to indeed support such a categorization. First the parapatric lake-stream systems both from Switzerland (Chapter 3) and Iceland (Chapter 6) as well as the phenotypically distinct clusters (Chapter 7) within Icelandic lakes derive each from a common genetic ancestor, i.e. a marine population. Applied on a larger geographic scale, we find that most studied freshwater systems are furthermore closer related to the marine population than to each other (Ólafsdóttir *et al.*, 2007a; b, Chapter 6 & 7, Lucek *unpublished data*). Consequently the studied stickleback systems fulfill the requirement of a common ancestry. Secondly, we find consistent and apparently repeatable patterns of parapatric phenotypic differentiation both among lake-stream systems in Switzerland (Chapters 2 & 3) and other European freshwater systems (Chapters 3 & 6). These match the predicted phenotype-environment relationships as inferred from other fish species (Barel, 1983; MacNeill & Brandt, 1990; Sandlund, et al., 1992a; Vonlanthen *et al.*, 2009) and stickleback systems in North America (Reimchen *et al.*, 1985; Schluter, 1993; McPhail, 1994; Thompson *et al.*, 1997; Robinson, 2000; Hendry *et al.*, 2002; Kaeuffer *et al.*, 2012) and Europe (Voje *et al.*, 2013; Ravinet et al., 2013b) with some notable differences especially for defense related traits (see Chapter 3 and below).

Another important criterion to infer adaptive radiation is trait utility (Schluter, 2000; but see the Introduction for a discussion). Evidence for the adaptive value of the observed phenotypic differentiation has mainly been indirectly inferred by correlating phenotypes with habitat and hence niche occupation (Chapter 2, 3 & 7), by the use of functional morphology (Chapter 5) and by comparing the observed habitat dependent changes with other published examples that test explicitly their adaptive value (Chapter 2-7). Especially the latter case may be problematic as it assumes a similar selective regime, adaptive potential and adaptive landscape among very different populations from different parts of Europe and even different continents (Chapter 3). Differences in the adaptive potential between distinct lineages of ancestral marine

populations have previously been found, where Pacific marine stickleback and their derived freshwater populations have a much increased standing genetic variation and hence adaptive potential as opposed to the Atlantic lineage (Jones *et al.*, 2012a,b), which is evolutionary much younger and derived from the Pacific (Orti *et al.*, 1994). Despite this different genetic background, freshwater populations that derive from both the marine Pacific and the Atlantic lineage evolve into ecological similar ecotypes (Reimchen *et al.*, 1985; Kaeuffer *et al.*, 2012; Moser *et al.*, 2012; Ravinet et al., 2013a; b; Chapter 2-4, 6). However, distinct phenotypes may underlie this parallel ecotype formation, potentially as a consequence of the different levels of standing genetic variation in the ancestral marine population and different genetic constraints (Berner *et al.*, 2010; Jones *et al.*, 2012b). For example, whereas limnetic feeding stickleback have significantly more gill rakers in comparison to benthic feeding populations in Northern America (Hagen & Gilbertson, 1972; Schluter, 1995; Robinson, 2000; Berner *et al.*, 2010), their European counterparts rather adaptive responses in gill raker length (Berner *et al.*, 2010; Ravinet *et al*., 2013b, see Chapter 3 for a comparison, but see Gross & Anderson, 1984). The adaptive landscape seems to furthermore differ between continents (Berner *et al.*, 2010; Ravinet *et al*., 2013b, Chapter 3), where habitat dependent phenotypic differentiation varies between continents for anti-predator related traits: On the one hand, it is predicted that selection leads to a reduction in the number of lateral plates during the colonization of freshwater (Colosimo *et al.*, 2004; Barrett et al. 2008). In Europe (Munzing, 1963; Lucek *et al.*, 2010; Lucek *et al.*, 2012b) and to a much smaller extent in North America (Reimchen *et al.*, 2013), marine-like phenotypes persist however in freshwater and may even experience positive selection in a marine-like lake environment (Chapter 3). Likewise differences in spine lengths appear to be more pronounced and parallel in European systems (Ravinet *et al.*, 2013b, Chapter 2, 3 & 6). Taken together, the observed phenotypic shifts in European stickleback as found throughout this thesis are likely to have an adaptive value as they mostly follow a predictable and repeatable direction, where phenotypic differences underlie changes in habitat use and niche partitioning as observed in other stickleback systems (Reimchen *et al.*, 1985; McPhail, 1994; Hendry *et al.*, 2002; Berner *et al.*, 2008; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b) and similarly differentiated fish species (Barel, 1983; MacNeill & Brandt, 1990; Sandlund, et al., 1992a; Vonlanthen *et al.*, 2009; Roesch *et al.*, 2013). Further experimental evidence is however needed (see Chapter 4 and Zeller *et al*., 2012a for an example).

Analogous to our inference of trait utility, rapid speciation was only indirectly assessed by the use of genetic markers (Chapters 2, 3, 6, 7). Here a significant degree of genetic differentiation was interpreted as an indication for a reduction in gene flow and hence the potential for non-random mating leading to reproductive isolation (see Hendry *et al.*, 2000; Koskinen *et al.*, 2002 for examples). The use of neutral genetic markers to infer ecological speciation was however questioned (Thibert-Plante & Hendry, 2010), because they can be affected by random processes such as drift, leading to the detection of false positive cases for ecological speciation. This applies especially when gene flow is low and divergence among all populations is high in the absence of divergent selection (Thibert-Plante & Hendry, 2010). Furthermore, differences at neutral

genetic markers may not necessarily reflect gene flow if a system is not at equilibrium. On the one hand, founder effects may cause increased genetic differentiation than expected at equilibrium. Therefore if two populations originate from two independent colonization events, founder effects or pre-existing genetic differentiation between the source populations could result in an underestimation of gene flow (Labonne & Hendry, 2010). On the other hand, if a large population splits into two in the absence of founder effects, the level of genetic differentiation at neutral genetic markers may be lower than at equilibrium and hence overestimating gene flow (Hendry *et al.*, 2000). Notwithstanding these theoretical limitations on the use of neutral genetic markers, the observed parapatric differentiation in Switzerland and Iceland are rather unlikely to represent false-positive cases of ecological speciation. First, populations within each system are most closely related to each other and systems are independent from one another (Chapter 6 & 7). Second, the observed genetic divergence is mirrored by the observed phenotypic divergence for traits that were previously suggested to experience habitat dependent divergent selection, which is furthermore paralleled in most studied systems. Standing genetic variation was in addition only mildly affected by potential founder events in Swiss systems in comparison to other European populations (Chapter 3). Founder events were however more pronounced in the Icelandic systems, but did not affect the phenotypic variation and hence the adaptive potential (Chapter 7).

Taken together, the observed patterns across our studied systems, geographical scales and time scales corroborate evidence for an onset of an adaptive radiation in stickleback (*sensu* Losos, 2010). The relative degree of progression differs however for each system, potentially as a combination of the genetic potential, ecological opportunity and the time for evolution, representing the early stages along the speciation continuum (Seehausen, 2009; Hendry *et al.*, 2009, Figure 4).

The role of phenotypic plasticity and adaptation from standing genetic variation during ecotype formation

Phenotypic evolution can be rapid (Thompson, 1998; Palumbi, 2001; Hairston *et al.*, 2005; Carroll *et al.*, 2007; Dlugosch & Parker, 2008; Hendry *et al.*, 2008), especially when measured over short time scales (Hairston *et al.*, 2005) and if selection acts on standing genetic variation (Facon *et al.*, 2006; Arnold *et al.*, 2008; Barrett & Schluter, 2008). The rate and extent of adaptive divergence is expected to depend on genetic and environmental constraints as well as the time for evolution to act (Nosil *et al.*, 2009); see Figure 2). Alternatively, phenotypes that match their environment may be produced instantaneously through adaptive phenotypic plasticity, wherein identical genotypes express different phenotypes in different environments (Gillespie, 1984; Keller & Taylor, 2008; Davidson *et al.*, 2011). Phenotypic plasticity and adaptation from standing genetic variation may both underlie the phenotypic evolution in single traits as well as shifts in higher dimensional trait space, affecting the underlying phenotypic (**P**) covariance matrix (Pigliucci *et al.*, 1999, Figure 2).

Adaptive evolution following rapid phenotypic changes may also be constrain by phenotypic plasticity as it shields the genome from the effects of selection and hence prevent the genetic fixation of distinct phenotypes (Price *et al.*, 2003; Ghalambor *et al.*, 2007). Over time, phenotypically plastic trait expression can itself become genetically fixed either through phenotypic canalization combined with genetic assimilation (Weinig, 2000; Yeh & Price, 2004; Crispo, 2007; Lande, 2009; Thibert-Plante & Hendry, 2011) or genetic accommodation, where selection acts on the reaction norm itself but may retain some plasticity (West-Eberhard, 2003). In both cases, successful genetic fixation depends among other factors on the strength of selection, the costs of maintaining plasticity, and the stability of the selection regime (West-Eberhard, 2003; Crispo, 2008; Lande, 2009; Thibert-Plante & Hendry, 2011).

Rapid phenotypic evolution occurs in most of our studied systems both during parapatric (Chapter 2-6; Table 1) and sympatric (Chapter 7) ecotype formation. We find that even evolutionary young systems can be as phenotypically differentiated as the much older systems that likely emerged shortly after the last glaciation period (Chapter 3), which is especially true for anti-predator related defense traits. Concomitantly, the **P** matrices of our studied systems change rapidly, where their leading eigenvectors (line of least resistance - p_{max}) differ already significantly between parapatric habitats ~50-100 years since the colonization of stickleback (Chapter 2). Habitat dependent differentiation in the **P** matric occurs moreover along shared trajectories for both defense and feeding related traits (Chapter 2 & 6). Several lines of evidence suggest that the observed phenotypic differentiation is at least partially genetically determined: i) In Lake Geneva, we still observe changes in population specific **P** matrices even a hundred years after the colonization, which is consistent with a gradual increase in differentiation over time (Chapter 2). Gradual evolution is also indicated over a longer time period in Chapter 6, where phenotypic convergent evolution is only reached among the oldest studied systems despite shared lines of least resistance. ii) Direct experimental evidence from Lake Constance suggests adaptive heritable differentiation together with a change in life history (Chapter 4; Moser *et al.*, 2012), where ecotypic differentiation in feeding related traits seems to be mainly genetically determined. iii) Phenotypic differentiation in anti-predator and feeding related traits occurs commonly between our studied parapatric ecotypes (Chapters 2-6) and between sympatric phenotypic clusters (Chapters 7). These categories are known to have a heritable component in stickleback (Peichel *et al.*, 2001; Hermida *et al.*, 2002; Chan *et al.*, 2009; Berner *et al.*, 2011), which may indicate that the rapid and parallel ecotype formation that we studied could be realized through selection on pre-existing standing genetic variation (Deagle *et al.*, 2012; Jones *et al.*, 2012b). For example, adaptive phenotypic changes in anti-predator related traits through selection on standing genetic variation were already observed over few (Kitano *et al.*, 2008; Le Rouzic *et al.*, 2011) or even just one generation (Barrett *et al.* 2008). Similarly genomic changes can occur over only a few years (Deagle *et al.*, 2012).

Plasticity has been commonly invoked to promote especially the initial colonization and subsequent adaptation during the colonization of a new environment (Weinig, 2000; Yeh & Price, 2004; Crispo, 2008; Lande, 2009;

Thibert-Plante & Hendry, 2011; Westley, 2011) as has been suggested for stickleback (Wund *et al.*, 2008; 2012). However, because we studied adaptive phenotypic differentiation in already established systems that were at least 50 years old, we cannot infer on the role of phenotypic plasticity during the earliest stages of ecotype formation. Yet, even after 140 years since introduction, we find evidence for a plastic component of adaptive phenotypic differentiation in our common garden like approach (Chapter 5). Moreover, the gradual changes in the **P** matrix could reflect the evolution of plasticity itself as suggested by theory (Lande, 2009; Draghi & Whitlock, 2012), where $p_{\max}$ based on plastic traits needs to reorientate itself. This seems to be rather unlikely the case in our studied systems as theory predicts this process to be fast, i.e. over less than 20 generations. In addition, parallelisms in $p_{\max}$ were observed across our entire studied time scale, ranging from 50 to 8000 year old freshwater systems (Chapters 2 & 6).

Taken together, both phenotypic plasticity and adaptation from standing genetic variation seem to underlie adaptive phenotypic evolution in postglacial stickleback systems, which is concordant with prior findings in this species (Day *et al.*, 1994; Hatfield, 1997; Berner *et al.*, 2011). The relative importance of either process depends however on the investigated trait combinations, where feeding and anti-predator related traits show stronger indications for selection on standing genetic variation, whereas differentiation in traits that are related to body shape and swimming performance are rather phenotypically plastic

Table 1: *Summary table for the genetic and phenotypic differentiation as well as the dimensionality of differentiation between parapatric lake-stream systems (Chapter 3 & 6) or intralacustrine phenotypic clusters (Chapter 7). The age, where known, is furthermore given for each lake system. The degree of genetic differentiation is defined as: +++ highly significant F_{ST} ($p < 0.001$) and evidence for genetic cluster based on STRUCTURE, ++ highly significant F_{ST} ($p < 0.001$) and significant F_{IS}, + non- or weakly significant F_{ST} and/or significant F_{IS}. Phenotypic differentiation is categorized as: +++ $p < 0.001$, ++ $p < 0.01$, + $p < 0.05$, - n.s, based on either P_{ST} for parapatric comparisons or MANOVA for sympatric comparisons. Dimensionality is measured as the number of distinct trait categories that are related to either feeding, anti-predator defense or body shape/swimming performance, where the number of "+" signs indicates the number of trait categories with at least one significant trait differentiation. Finally, differences in the line of least resistance (p_{max}) are given as the number of trait categories for which the parapatric p_{max} differs. Empty cells indicate cases for which no data was available.*

			Parapatric lake-stream differentiation				Sympatric intralacustrine differentiation		
	System	Age [yrs]	Genetic differentiation	Phenotypic differentiation	Dimensionality	p_{max}	Genetic differentiation	Phenotypic differentiation	Dimensionality
Switzerland	Bern	50-80	+	-	+	-			
	Biel	50-80	+	+++	+++				
	Geneva	140	+++	+++	+++	++			
	Constance	140	+++	+++	+++	++			
Iceland	Hraunsfjördur	~50	+	-	+++	+	+	-	-
	Apavatn	?[1]					-	+++	++
	Flódid	?[1]					-	-	+
	Mjóavatn	?[1]					-	++	+
	Galtaból	?[1]					-	-	+++
	Frostastavatn	?[1]					-	+++	+++
	Mómelar	?[1]					+	+++	+++
	Mývatn	~2500	++	+++	+++	+	++	+++	+++
	Thingvallavatn	~8000	+	+++	+++	-	++	+++	+++

[1] These lakes emerged historically after the last glaciation period.

Along the speciation continuum: From invasion to diversification and beyond

Ecological speciation is thought to proceed along an evolutionary continuum from intraspecific variation with a single phenotypic and genotypic cluster to bimodal distributions of phenotypic or genetic clusters with varying levels of reproductive isolation, and eventually two discrete and fully isolated species (Seehausen et al., 2008a; Hendry, 2009; Seehausen, 2009; Nosil *et al.*, 2009; Nosil, 2012), Figure 4). Along the continuum, geographic and/or ecological clines in gene frequencies of adaptive alleles and adaptive phenotypic variation should similarly evolve from a rather shallow to a steep cline (Endler, 1977; Nosil *et al.*, 2009), Figure 4). The progression along this continuum can be interrupted or reversed and consequently speciation may remain incomplete for a considerable amount of time. If this process operates in spatially structured metapopulations, variation in the degree of progression and reversal along the continuum is expected to result in pairs of ecologically differentiated populations at different stages (Seehausen, 2009; Nosil et al., 2009). Although empirical studies that place populations and species to the different stages along a speciation continuum within the same taxon have recently emerged (Nosil, 2012; Feder et al., 2012), including examples from invertebrates (*Timema* walking-stick insects Nosil & Sandoval, 2008, pea aphids Peccoud et al., 2009, Heliconius butterflies Nadeau et al., 2013) and fishes (Pundamilia cichlids Seehausen et al., 2008a, threespine stickleback Hendry *et al.*, 2009), the comparative investigation of the very early stages of the speciation continuum are yet rare.

The evolution of genetic differentiation between differentially adapted ecotypes and species also falls along the speciation continuum, ranging from no to strong genetic differentiation across genomes (Feder et al., 2012). At its early stages, divergent selection is predicted to lead to the fixation of different adaptive alleles between distinct populations, whereas for selectively neutral markers, gene flow is still abundant. Genomic regions under divergent selection may then increase in size with time as physically linked gene regions experience similar divergent selection through divergent hitchhiking combined with a reduction in gene flow (Feder *et al.*, 2012; Nosil, 2012). As speciation proceeds, intrinsic prezygotic isolation may evolve, reducing interspecific gene flow and increasing genomic divergence.

The patterns of phenotypic and genotypic differentiation observed among our studied systems are consistent with the early stages of along the speciation continuum (Table 1). The evolutionary youngest systems (Bern & Hraunsfjördur) show the least genetic and phenotypic structure between parapatric habitats. As speciation progresses, we find increased levels of phenotypic differentiation as well as an increase in its dimensionality both for parapatric (Chapter 6; Table 1) and sympatric (Chapter 7) cases. Increased dimensionality seems on the one hand to be driven by the time that is available for evolution to act (Chapter 6 & 7) and on the other hand by ecological opportunity potentially combined with intraspecific competition (Chapter 7). This follows theoretical predictions for intraspecific diversification in heterogeneous habitats or along environmental gradients, leading to the occurrence and existence of distinct phenotypes (Doebeli & Dieckmann, 2003; Leimar et al., 2008; Débarre, 2012). Especially frequency dependent competition along an environmental gradient has the potential to cause the formation of distinct phenotypes in space (Doebeli & Dieckmann, 2003). This can furthermore occur in sympatry and in the presence of gene flow, where the degree of phenotypic overlap further depends on the steepness of the environmental gradient (Leimar et al., 2008) as we observe for sticklebacks from different Icelandic lakes (Chapter 7).

The patterns observed for phenotypic differentiation stay in contrast to the levels of genetic differentiation. For parapatric lake-stream ecotypes, genetic differentiation was highest in lakes Constance and Geneva (Chapter 6). In the latter case, we additionally find evidence for a genetic cline due to selection on the *Eda* gene (Chapter 2). In contrast, parapatric genetic differentiation in the two older Icelandic lakes Mývatn and Thingvallavatn was weaker. Overall the level of genetic differentiation was significantly correlated with the environmental gradient (Chapter 6). Thus, the difference between phenotypic and genetic differentiation may reflect the very early stage along the speciation continuum, where using primarily neutral markers, neutral genetic differentiation is expected the rather weak as selection acts only on a small fraction of the genome (Feder *et al.*, 2012). Genetic structure in such cases may only become apparent if many phenotype-linked markers are studied that experience divergent selection as it was found for Lake Thingvallavatn (Ólafsdóttir & Snorrason, 2009), where the potential for reproductive isolation has been previously suggested (Ólafsdóttir *et al.*, 2006). Neutral genetic differentiation may however build up over time as it is indicated for sympatric

stickleback ecotypes in the two larges Icelandic lakes (Chapter 7), suggesting an advanced stage of ecological speciation (Hendry, 2009; Seehausen, 2009; Nosil, 2012; Feder *et al.*, 2012).

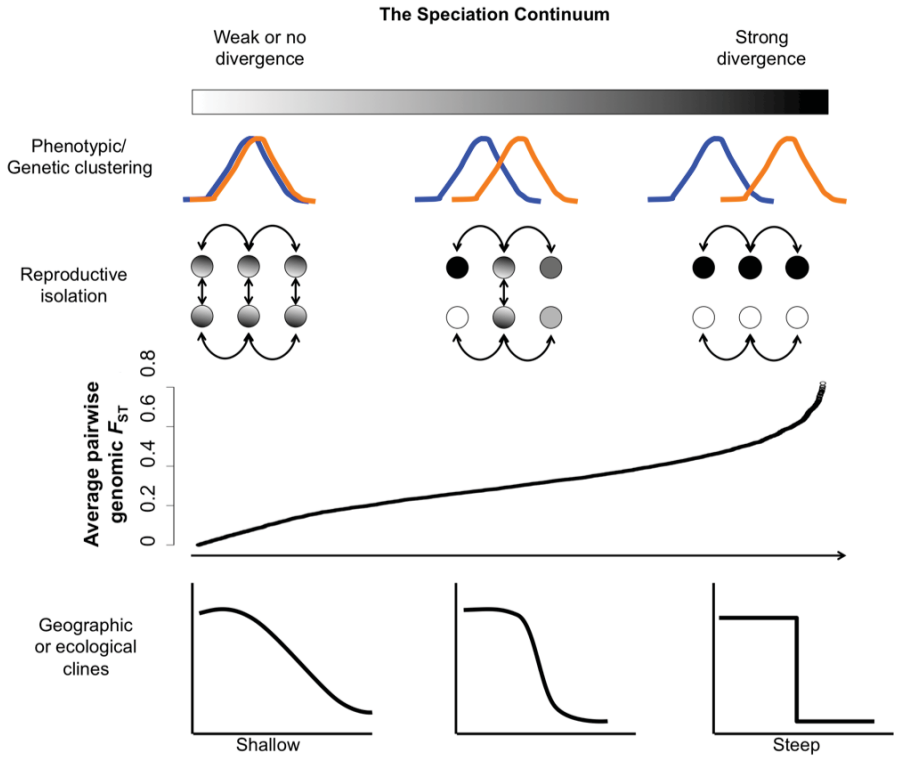

Figure 4: *The continuous speciation process. a) Progression along the speciation continuum is accompanied by increased reproductive isolation and phenotypic and genetic divergence, ranging from different populations to ecotypes of the same species to the formation of distinct and specialized species. Arrows represent mating between individuals. (Modified from* (Nosil *et al.*, 2009)*). Progression along the continuum leads to an increase in average pairwise genomic divergence (*F_{ST}) between distinct ecotypes and species*. Lastly, the steepness of geographic and/or ecological clines for adaptive allele frequencies may change by becoming increasingly steeper with progression along the continuum.*

Some missing pieces and potential avenues for future research

Although this thesis improves our understanding of the evolutionary processes that underlie biological invasions and contemporary ecotype formation and ultimately the formation of species, many questions remain unanswered or emerge from it and call for future investigation. Especially new technological advances, such as next generation sequencing will allow to further broaden our knowledge on how species are formed and maintained (Rice *et al.*, 2011; Feder *et al.*, 2012). A major concern is still to identify and understand the actual agents of selection that lead to the observed and repeated patterns in our studied systems. For example, while anti-predator related phenotypic traits and their underlying genes seem to experience divergent selection (Zeller *et al.*,

2012a), classical hypotheses (Reimchen, 1980; 1994) fail to explain the observed phenotypic differentiation in Switzerland (Zeller *et al.*, 2012b) and Iceland (Lucek *et al*., 2012b). Because several projects that partially build on the results presented in this thesis are currently performed, investigating i) the genomic aspects of the repeated ecotype formation in Swiss sticklebacks (Marques *unpublished data*), ii) the role of parasites as selective agents (Anaya-Rojas *in preparation*) and iii) the effect induced by the different stickleback ecotypes on the ecosystem (Aebischer *unpublished data*), I will only outline some of the exciting additional avenues of research that emerged from carrying out this thesis.

Flipping the coin - Adaptive diversification vs. adaptive sexual dimorphism

Throughout this thesis, morphological and in most cases ecotypic diversification could be found that is associated with the colonization of different parapatric habitats (Chapters 2-6) and even in sympatry (Chapter 7). Whereas it was generally suggested that the observed pattern is consistent with the formation of phenotypically distinct ecotypes, alternative evolutionary outcomes may be possible, such as the formation of adaptive sexual dimorphisms (Slatkin, 1984; Bolnick & Doebeli, 2003; Van Dooren *et al.*, 2004; Cooper *et al.*, 2011). Indeed, theory suggests that adaptive diversification and adaptive sexual dimorphisms represent two sides of the same ecological coin (Bolnick & Doebeli, 2003). Ecological sexual dimorphism can be caused by disruptive selection due to frequency-dependent intraspecific competition (Slatkin, 1984; Bolnick & Doebeli, 2003; Rueffler *et al.*, 2006) and sexual conflict (Cox & Calsbeek, 2009). Further phenotypic diversification can take place by disruptive selection on female preferences, leading to a higher number of phenotypic clusters and eventually speciation (Van Dooren *et al.*, 2004). Furthermore, adaptive diversification and adaptive dimorphisms may occur at the same time, leading to multiple ecologically differentiated clusters (Cooper *et al.*, 2011).

However, in cases where adaptive ecological sexual dimorphisms occur, ecological speciation could be absent or incomplete, because females would still mate with phenotypically divergent males and hence mitigate divergent selection. This has been found in multiple lake dwelling stickleback populations in Alaska (Bolnick & Lau, 2008). Here the authors found a weak trend towards disruptive selection between sexes among 14 lakes due to intraspecific competition for alternative resources. Furthermore they found the strongest disruptive selection in lakes of intermediate sizes (0.2-0.4 km^2), where the amount of littoral and pelagic prey is balanced. In comparison to the studied systems in Iceland and Switzerland in this thesis (Chapter 7), lakes are larger than this size range, hence under the assumption of a similar adaptive landscape, the outcome should be less balanced than what we observed – fewer phenotypic clusters or a strong bias towards one dominating cluster would be expected. Only if ecologically divergent traits become sexually selected ("magic traits", Gavrilets, 2004), strong selection would lead to reproductive isolation. Although the potential for sexual dimorphisms *within* a population, where sexes differ phenotypically is indicated in some of the studied systems in this thesis (Lucek *personal observation*, Kristjánsson *et al.*, 2002a) a formal test is still needed. Quantifying the potential for adaptive sexual dimorphisms may help to

determine why in some cases populations show a higher trait variation among individuals than in others (e.g. Chapter 3, Figure 8). Sexual dimorphisms may especially be promoted in large lakes where fish have a potamodromous life style. Here males stay for a long time period on the breeding ground and start feeding on locally available prey whereas females only visit to spawn and migrate back to the lake.

The role of interspecific competition as an evolutionary driver

Many studies mainly focused on adaptive radiation of a single taxon in a specific location, with only few studies comparing similar radiations of the same taxa on a broader geographic scale (e.g. Losos *et al.*, 1998; Hudson *et al.*, 2011; Wagner *et al.*, 2012). Yet replicated adaptive radiations of different taxa may occur in coexistence and hence sympatry and may either promote the diversification of each other by providing a potential resource for one taxon or impede diversification of one taxon through interspecific competition. Intraspecific diversification and the increase of adaptive diversity may furthermore affect the entire ecosystem and subsequently the present community structure (Vellend *et al.*, 2007; Ehrenfeld, 2010). Overall our understanding of the role of interspecific competition and coexistence in facilitating or constraining adaptive diversification remains incomplete and represents a major research gap in evolutionary ecology (Nosil, 2012).

The possibility to study such processes in the systems investigated in this thesis is indeed present. Stickleback populations from both Switzerland and Iceland are known to each coexist with at least one described adaptive radiation: whitefish *Coregonus* sp. in Switzerland (Hudson *et al.*, 2011) and arctic charr *Salvelinus* sp. in Iceland (Sandlund, *et al.*, 1992a; Gislason *et al.*, 1999). In both cases distinct ecotypes and life stages of different species may ecologically overlap and hence constrain the potential for adaptive divergence in each species. On the other hand the presence of stickleback may promote the formation of a predatory ecotype in the other species as has been indicated for arctic charr (Riget *et al.*, 2000). Albeit the distinct interspecific interactions may proof difficult to disentangle in the relatively species rich communities of Swiss lakes, especially the Icelandic lakes with only up to three species being present provide a model-like natural setting to investigate interspecific interactions. A first attempt to compare coexisting sticklebacks and arctic charr ecotypes from lake Thingvallavatn is outlined in Box 3. Further ecological and genomic studies are however needed to assess for example the strength of divergent selection between ecotypes of a single radiation in the presence of other species.

Understanding the epigenomics of speciation

The field of epigenetics and most recently epigenomics has gained increasing interests for evolutionary biologists as it allows to understand the genomic aspects of phenotypic plasticity (Aubin-Horth & Renn, 2009; Laird, 2010; Ekblom & Galindo, 2011). For example, the advance of next generation sequencing technologies opens an interesting opportunity to gain insights on the methylation patterns across entire genomes (Cokus *et al.*, 2008; Bock, 2012).

Such methylation patterns seem to be to some extend heritable and potentially linked to phenotypic plasticity, where genomic regions that are methylated are less transcribed than unmethylated regions (Richards *et al.*, 2010). In the case of the threespined stickleback, outlined in this thesis and elsewhere (e.g. Wund *et al.*, 2008), plasticity seems to be a main driver for a part of the potentially adaptive phenotypic variation among populations (e.g. Chapter 5), which may or may not become genetically fixed over time through canalization (see Chapters 2 & 6 for a discussion). Epigenomic approaches could therefore be further employed to actually test for canalization along the temporal gradient investigated here (Chapter 6) and more generally along different speciation gradients. A simple prediction would be that the overall methylation along the genome should increase with evolutionary time as a population becomes less plastic and increasingly specialized. Similarly a combination of genomic and epigenomic approaches could be used to distinguish between an increase in phenotypic plasticity or standing genetic variation that lead to a higher phenotypic variation in genetically admixed populations in Swiss sticklebacks (Lucek *et al.*, 2010, Lucek *et al. manuscript*) or other cases of species admixture and hybridization (Seehausen, 2004; Stelkens *et al.*, 2009; Stelkens & Seehausen, 2009a; b).

Chapter 2

Contemporary ecotypic divergence during a recent range expansion was facilitated by adaptive introgression

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Manuscript

Abstract

Contemporary adaptive phenotypic evolution during range expansion associated with colonization of contrasting habitats has been documented in several taxa. The evolutionary trajectories that underlie such phenotypic divergence have however only rarely been investigated. A strong candidate for contemporary adaptive divergent evolution within an invaded range is the threespine stickleback in the Lake Geneva region of central Europe, which was introduced only about 140 years ago. Since then, it has undergone both a range and a niche expansion, now forming phenotypically differentiated parapatric ecotypes that occupy the pelagic zone of the large lake on the one hand, and little inlet streams on the other hand. By comparing museum collections with contemporary population samples, we aim to reconstruct the evolution of habitat dependent phenotypic divergence through time. Using genetic data from modern samples we infer the underlying invasion history. We find consistent parapatric divergence in phenotypes between the lake and stream habitats through time. Especially anti-predator related traits show consistent habitat dependent divergence, with the magnitude of differentiation increasing through time. This suggests a selection regime that was stable through much of the time since colonization, where the recently increased phenotypic differentiation likely results from secondary gene flow from a distantly related lineage outside the Lake Geneva region that introduced new alleles of major phenotypic effects. Our results suggest that divergent selection between habitats has quickly lead to some phenotypic differentiation among populations inhabiting these habitats, but that an increase in genetic variation through hybridisation promoted further divergence.

Introduction

Natural selection that favours different phenotypes in different habitats during range expansion and colonization of new habitats can lead to rapid contemporary divergent evolution and to the formation of ecotypes that are distinct from the ancestral source population and distinct from each other, potentially initiating ecological speciation (Schluter, 2000; Carroll *et al.*, 2007; Berner, 2009; Bacigalupe, 2009; Losos, 2010; Badyaev, 2010; Eroukhmanoff & Svensson, 2011). Such rapid ecotype formation has been observed in several biological invasions (Elton, 1958; Baker, 1980; Hendry *et al.*, 2000; Koskinen *et al.*, 2002; Facon *et al.*, 2006; Chapuis *et al.*, 2008). The evolutionary trajectories that underlie phenotypic divergence have however only rarely been investigated due to the common lack of historical data to compare the contemporary populations to (but see Hairston *et al.*, 1999; Badyaev, 2010; Calsbeek *et al.*, 2011). Phenotypic evolution can be fast (Thompson, 1998; Palumbi, 2001; Hairston *et al.*, 2005; Carroll *et al.*, 2007; Dlugosch & Parker, 2008; Hendry *et al.*, 2008), especially when measured over short time scales (Hairston *et al.*, 2005) and if selection acts on standing genetic variation (Facon *et al.*, 2006; Arnold *et al.*, 2008; Barrett & Schluter, 2008). The rate and extent of adaptive divergence is expected to depend on genetic and environmental constraints as well as the time for evolution to act (Nosil *et al.*, 2009). Alternatively, phenotypes that match their environment may be produced instantaneously through adaptive phenotypic plasticity, wherein identical genotypes express different phenotypes in different environments (Gillespie, 1984; Keller & Taylor, 2008; Davidson *et al.*, 2011). Plasticity may however need to evolve itself in response to novel selection regimes, which can theoretically occur over a few generations (Lande, 2009).

Heritable phenotypic evolution is thought to proceed along so called "lines of least resistance" or $\boldsymbol{g}_{max}$ that can be quantified as the leading eigenvector of the genetic variance-covariance matrix **G**, which summarizes the additive genetic contribution to the variances and covariances among phenotypic traits (Lande, 1979; Schluter, 1996; see Steppan *et al.*, 2002 for a review). Biologically, this axis comprises most of the heritable phenotypic variation and genetic constraints, shaped by mutation, selection and drift within a population (Marroig & Cheverud, 2005). Evolution is predicted to be biased towards the direction of this leading axis, imposing genetic constraints on adaptation over short timescales unless $\boldsymbol{g}_{max}$ is aligned with the direction of selection (Lande, 1979; Arnold *et al.*, 2008). Over longer time, selection may change the orientation of $\boldsymbol{g}_{max}$ towards the new adaptive optimum (Bacigalupe, 2009; Badyaev, 2010; Eroukhmanoff & Svensson, 2011). Mutation and drift as well as gene flow may furthermore alter the **G** matrix and hence $\boldsymbol{g}_{max}$ over time (Guillaume & Whitlock, 2007; Chapuis *et al.*, 2008).

In the absence of quantitative genetic data, the **G** matrix can be approximated by the **P** matrix, which is based on phenotypic data from wild populations (Arnold *et al.*, 2008). Here, **P** is defined as the combination of the genetic and environmental covariance matrices, i.e. **G** + **E** (Lande, 1979; Arnold & Phillips, 1999), which may interact (**G** x **E**; Falconer, 1989). Consequently, **P** matrices may include phenotypically plastic traits that are differentially expressed between different environments (Pigliucci *et al.*, 1999). The leading

eigenvector of a **P** matrix ($\boldsymbol{p}_{\mathbf{max}}$) may therefore serve as an overall measure of phenotypic variation observed in the wild, accounting for both genetic and environmental effects. Once a population experiences a new selective regime, $\boldsymbol{p}_{\mathbf{max}}$ may begin to be redirected towards the new adaptive peak (Arnold *et al.*, 2008). During adaptation towards a new adaptive optimum, the angle between the ancestral $\boldsymbol{p}_{\mathbf{max}}$ and a derived $\boldsymbol{p}_{\mathbf{max}}$ consequently increases with time (Schluter, 1996). If new adaptive phenotypes are achieved mainly by phenotypic plasticity, $\boldsymbol{p}_{\mathbf{max}}$ should redirect rapidly within one or over just very few generations (Lande, 2009; Draghi & Whitlock, 2012), whereas a gradual increase over many generations is expected if phenotypic evolution is mainly heritable (Schluter, 1996). Likewise the shape of a **P** matrix, estimated by its eccentricity, i.e. the ratio of the two leading eigenvectors, can change depending on the selective regime acting on it. For example, increased directional selection leads to an increase in eccentricity (Jones *et al.*, 2003). Therefore, studying time series of population-based **P** matrices in distinct environments allows investigating the major trajectories along which phenotypic evolution occurs.

The threespine stickleback (*Gasterosteus aculeatus* species complex) has become a model system in speciation research (McKinnon & Rundle, 2002). Throughout the Northern Hemisphere stickleback repeatedly colonized freshwater systems from the Sea shortly after the last glacial period ended about 12,000 years ago. During this process, they have adapted to different habitats such as lakes and streams and formed different ecotypes and species (McKinnon & Rundle, 2002). In Switzerland, stickleback were historically restricted to a small region in the northern part of the country north of the Jura mountains but got introduced at several other sites about 140 years ago. Introduced populations derived from several evolutionary different European lineages, belonging to two nominal species, *Gasterosteus aculeatus* in the NE of Switzerland and *G. gymnurus* in the SW and NW (Lucek *et al.*, 2010). Because this taxonomical distinction may be ambiguous, we will refer to them as the *G. aculeatus* species complex (Bell & Foster, 1994). Since colonization, both lineages have undergone a massive range expansion, forming a broad hybrid zone across Switzerland. This range expansion was associated with considerable niche expansion, with populations having colonized different habitats including little streams and the pelagic zone of large lakes, forming phenotypically differentiated ecotypes (Roy *et al.*, 2010; Berner *et al.*, 2010; Lucek *et al.*, 2012a; Lucek *et al.*, 2013).

A strong candidate for contemporary ecotype formation has been identified in the Lake Geneva region within the Rhone drainage of Switzerland, where individuals from the lake differ from stream fish in both feeding related morphology and in defence related phenotypes, e.g. the number of bony lateral plates along an individual's flank (Gross, 1977; Lucek *et al.*, 2010; Roy *et al.*, 2010; Berner *et al.*, 2010; Lucek *et al.*, 2013). However, through most of the population history since colonization only individuals with few lateral plates (so called low plated morphs) were documented in the Lake Geneva region, consistent with expectations for *G. gymnurus* (Lucek *et al.*, 2010 and references therein). Yet in other parts of Switzerland, especially in the Lake Constance region, populations exist that have been predominantly fully plated, i.e. have plates contiguously along the whole body (consistent with expectations for *G.*

aculeatus). Lateral plate morph polymorphisms are known to have a simple, almost Mendelian genetic architecture which is linked to the *Ectodysplasin* (*Eda*) locus (Colosimo *et al.*, 2004; 2005). Both the alternative plate phenotypes as well as the genotypes themselves are known to experience divergent selection between distinct habitats such as lakes and streams (Reimchen, 1994; Barrett et al. 2008; Barrett, 2010).

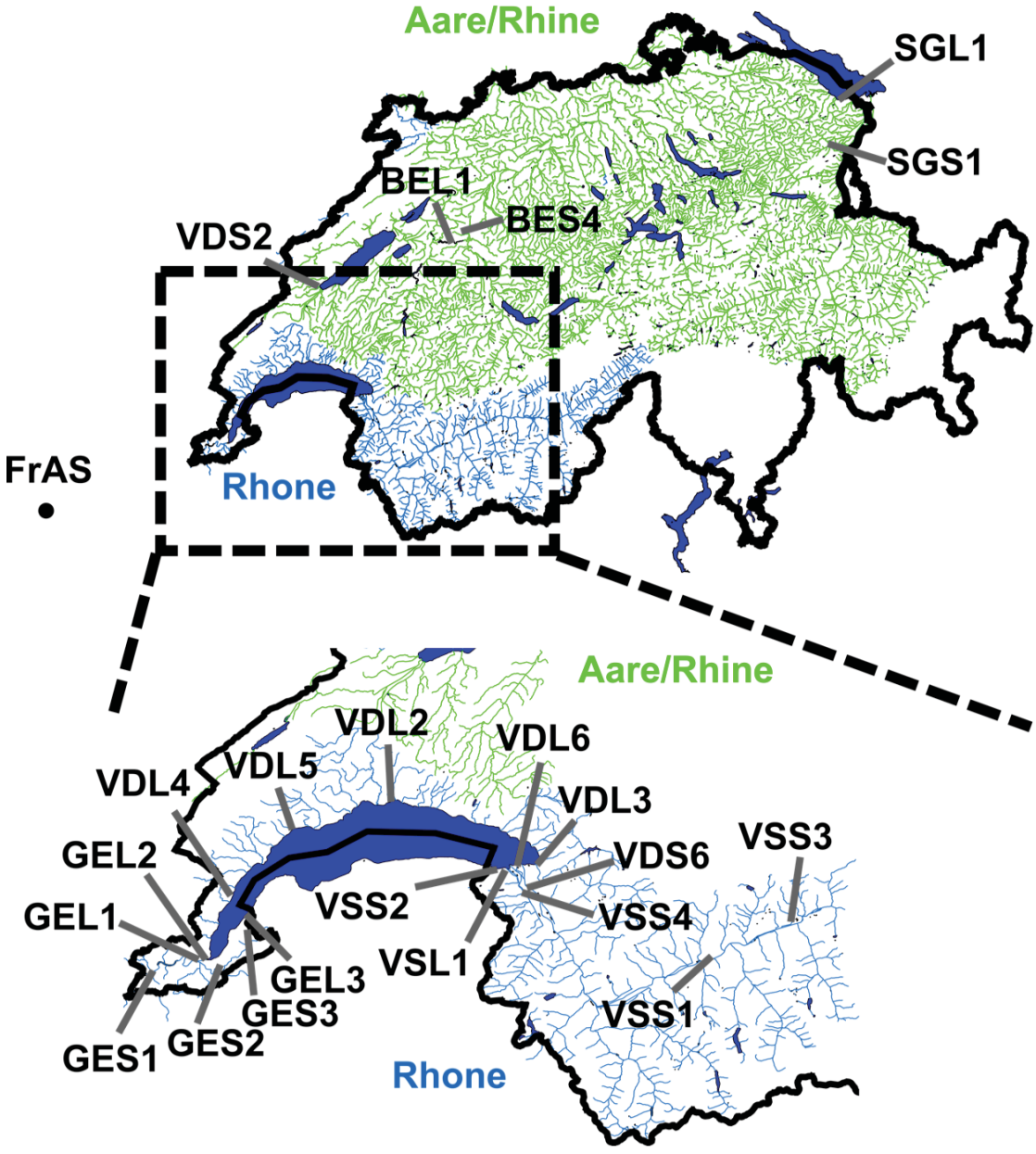

Figure 1: *Map of Switzerland (© SwissTopo 2012) with all Swiss sampling sites indicated (see table 1 for details). The colors of waterways represent the two major drainage systems of Switzerland, i.e. Rhone and the Aare/Rhine system.*

By comparing the phenotypic (**P**) covariance matrices of historical museum and contemporary collections from several populations in both streams and the lake, we ask if we can demonstrate gradual changes through time towards the currently observed phenotypic differentiation of lake and stream

ecotypes, or if differentiation was already complete by the time of our earliest samples. Because the underlying selective regime may differ between different trait combinations, we separately analyse functionally different trait categories, related either to anti-predation defence or to feeding ecology (Reimchen, 1994; Walker, 1997; Albert *et al.*, 2008). We predict that the $\boldsymbol{p}_{\max}$ of different populations from the same habitat should align independently of time if habitat dependent ecotypic differentiation was completed quickly, i.e. in the first few decades after colonization. Alternatively, more gradual evolution predicts that we can see the angle between $\boldsymbol{p}_{\max}$ and the common line of least resistance increasing over time. In a second step, we infer the genetic history of this biological invasion from contemporary population samples and test if the contemporary phenotypic differentiation between ecotypes was a result of repeated colonization and secondary contact, where distinct habitats may have been colonized by genetically and phenotypically different colonizing lineages (Taylor & McPhail, 2000), or rather reflects *in situ* evolution. Finally, we test if both lateral plates and the *Eda* locus itself show patterns of habitat dependent selection and may reflect patterns of adaptive introgression (Rieseberg, 2011; Pardo-Diaz *et al.*, 2012).

Material and methods

Sample collection

Eight historical population samples from 1921-1979 of ≥ 20 individuals each were available from the Natural History Museums in Geneva and Lausanne (Table 1), with a total of 438 individuals originating from both lake and stream habitats. None of the museum specimens was available for DNA extraction. In addition, a total of 659 individuals from sixteen contemporary populations within the Lake Geneva system were collected between 2007 and 2013 using hand nets and minnow traps (Figure 1). Hereafter we refer to these two categories of samples as either of "historical" or "contemporary" origin. Some contemporary populations were collected during an earlier study of the invasive range of sticklebacks in Switzerland (Lucek *et al.*, 2010). Further effort was then made to sample the same sites as those from which historical samples were available. However, only at five of these sites sticklebacks could be observed and collected (Table 1). Because only juvenile individuals were be obtained at the GEL3 site that do not have their plates fully developed (Bell, 1981), this population was only included for the *Eda* related analyses. Populations were assigned to be either lake or stream dwelling based on the habitat where they were sampled. Contemporary individuals were preserved in 70% ethanol after taking a fin clip, which was stored in absolute ethanol for genetic analysis.

Table 1: *Location, year of collection, sample size (N) for individuals used for morphology, microsatellites (µsat) and Stn382, coordinates of sampling site, habitats and collection ID of the respective natural history museum (NHMG=Geneva, NHML=Lausanne). Population names consist of two letters for Swiss sites or three letters for non-Swiss sites, followed by the habitat type (L, lake; and S, stream) and by a serial number.*

	Historical					***Contemporary***					
Population	**Origin**	**Habitat**	**Year**	**$N_{Morphology}$**	**ID**	**Year**	**$N_{Morphology}$**	**$N_{\mu sat}$**	**N_{Stn382}**	**Coordinates**	
VSS3	Geneva	Stream	1921	84	NHMG-816	2009	96	26	96	46°16'45" N	7°30'55" E
VDS6	Geneva	Stream	1958	36	NHML-163	2012	32	32	32	46°20'52" N	6°54'38" E
			1964	28	NHML-4216, -4772						
GES2	Geneva	Stream	1979	35	NHMG-2063, -2064	2009	42	32	42	46°11'48" N	6°11'30" E
VSS1	Geneva	Stream	-	-		2007	102	30	96	46°12'50" N	7°18'53" E
VSS2	Geneva	Stream	-	-		2007	59	32	59	46°23'07" N	6°51'30" E
GES1	Geneva	Stream	-	-		2008	36	32	36	46°10'47" N	6°00'32" E
GES3	Geneva	Stream	-	-		†2009	6	6	6	46°18'09" N	6°14'51" E
VSS4	Geneva	Stream	-	-		†2012	48	29	48	46°20'20" N	6°53'20" E
VDL4	Geneva	Lake	1967	20	NHML-6520	2010	30	32	23	46°18'18" N	6°10'55" E
VDL6	Geneva	Lake	1967	70	NHML-6465	2012	37	30	32	46°23'45" N	6°53'18" E
GEL1	Geneva	Lake	1978	34	NHMG-1592	-	-	-	-	46°12'14" N	6°08'02" E
GEL2	Geneva	Lake	1979	131	NHMG-2009	-	-	-	-	46°12'10" N	6°07'54" E
VDL2	Geneva	Lake	-	-		2008	40	30	40	46°31'02" N	6°34'41" E
VDL3	Geneva	Lake	-	-		2010	34	19	32	46°23'38" N	6°55'18" E
VDL5	Geneva	Lake	-	-		2010	30	16	30	46°27'12" N	6°20'11" E
VSL1	Geneva	Lake	-	-		†2012	36	28	37	46°23'09" N	6°51'29" E
GEL3	Geneva	Lake	-	-		2013	-	29	30	46°17'57" N	6°14'32" E
FrAS	France	Stream				2012	32	32	32	45°58'04" N	5°17'40" E
VDS2	Neuchatel	Stream				2008	-	31	-	46°38'30" N	6°37'36" E
BEL1	Bern	Lake				2007	-	30	-	46°57'59" N	7°21'09" E
BES4	Bern	Stream				2008	-	28	-	46°59'31" N	7°24'42" E
SGL1	Constance	Lake				2007	-	30	-	47°29'08" N	9°32'38" E
SGS1	Constance	Stream				2007	-	28	-	47°19'33" N	9°34'41" E

† Only the number of lateral plates was counted.

Morphological analysis

Sixteen linear morphological traits were measured to the nearest 0.01 mm using a digital calliper. These traits were either related to anti-predator defense, feeding ecology or general body shape and swimming performance (Mori & Takamura, 2004; see Figure 2). In addition, the number of lateral plates on the left flank was counted using a dissection microscope. Because all linear traits were significantly correlated with standard length (results not shown), a local size correction was applied. Each trait was first scaled by the mean within each population as suggested by Houle (1992). Subsequently, a linear regression of each trait against SL was performed separately for each population, retaining the residuals for further analysis (Berner et al., 2010b).

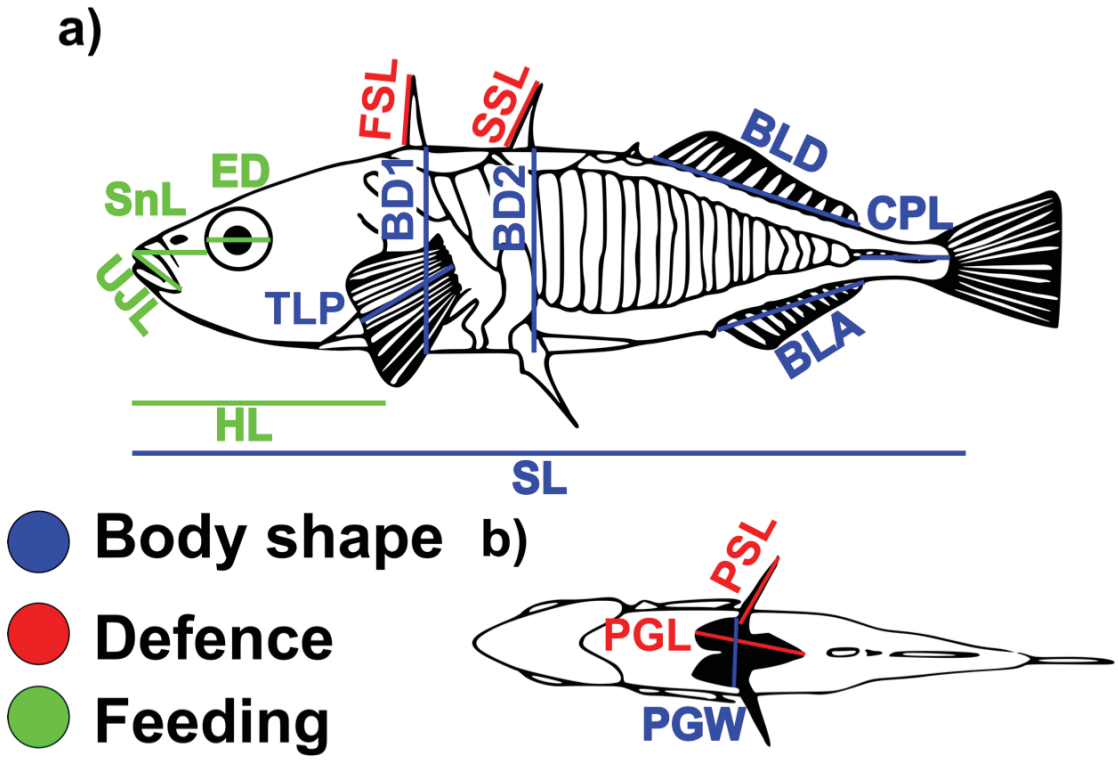

Figure 2: *Linear measurements that were measured either on the left flank a) or on the ventral side b) for each specimen. Traits were either linked to anti-predator defence (red), feeding ecology (green) or body shape and swimming performance (blue). Trait abbreviations are as follow: FSL -length of the first dorsal spine; SSL - length of the second dorsal spine; PSL - length of the pelvic spine; PGL - length of the pelvic girdle; HL - head length; UJL - upper jaw length; SnL - snout length; ED - eye diameter; SL - standard length; PGW - width of the pelvic girdle; BD1 - body depth measured after the first dorsal spine; BD2 - body depth measured after the second dorsal spine; caudal peduncle length; BLA - basal length of the anal fin; BLD - basal length of the dorsal fin; TLP - total length of the pelvic fin*

Pairwise phenotypic comparisons were performed for the five sites where both historical and contemporary population samples were available

(GES2, VDL4, VDL6, VDS6, VSS3; Table 1). The pairwise phenotypic divergence over time was then estimated using pairwise P_{ST}, an analogue to Q_{ST}, based on phenotypic data from wild individuals following Kaeuffer et al. (2012). P_{ST} was based on the first principal component (PC) axis of either all phenotypic traits combined, or separately for defense and feeding related phenotypic traits. P_{ST}s and their significance levels were calculated using a resampling approach with 1000 replicates. Divergence of the evolutionary trajectory over time within a sampling site was further estimated as the angle between the $\boldsymbol{p_{max}}$, separately calculated for each sampling event within a site. Significance was estimated using a bootstrap resampling procedure with 1000 replicates following Berner (Berner, 2009). In addition, the morphospace occupied was estimated as the ellipse size of the 95% confidence interval based on the two leading PC axes. Eccentricity of the covariance matrix, was calculated as the ratio between the eigenvalues of the two leading PC axes. All calculations were performed using either all linear traits combined or separately for feeding and defence related traits.

The multivariate direction of phenotypic divergence along both the habitat and time axis was compared by calculating the angles between the leading eigenvectors ($\boldsymbol{p_{max}}$) of each sampling event and site against the overall $\boldsymbol{p_{max}}$, where all individuals were pooled (Schluter, 1996). The significance of each comparison was tested using 1000 bootstrap replicates. Obtained angles were then tested for a significant association with either *habitat* (lake and stream) or *time* (historical and contemporary) using linear models, where the best fitting model was determined using a stepwise backward procedure.

A linear mixed effects model was used to test if the lateral plate counts statistically differ between historical and contemporary sites (i.e. *time*) as well as between lake and stream sites (i.e. *habitat*). This model assumed a Poisson distribution and corrected for multiple sampling events from the same site. The best fitting model was determined using a stepwise backward procedure. Significant differences among groups were determined using post hoc *t* tests. Habitat dependent differentiation in lateral plate counts was furthermore tested separately among historical and contemporary individuals.

Genetic analysis

In total, 403 individuals from 16 contemporary sites within the Rhone drainage, including FrAS were genotyped at nine microsatellites (Table 1), of which three markers (Stn26, Stn96, Stn130) are putatively linked to QTLs related to spine lengths (Peichel *et al.*, 2001). In addition, 147 individuals from five populations within the invasive range of sticklebacks in Switzerland outside the Rhone drainage system were included to test for potential gene flow between drainage systems. DNA for all individuals was extracted using a 10% Chelex solution, following the manufacturers protocol (Biorad, California, USA). All microsatellites were amplified in one multiplex kit following Raeymaekers *et al.* (2007). Detailed information on the multiplexing setup and the PCR protocol are provided as supplementary methods. Alleles were visualized on an ABI 3130XL and scored with GeneMapper 4.0 (Applied Biosystems, Switzerland).

Microsatellite based pairwise F_{ST} values, were calculated with GENODIVE 2.0B22 (Meirmans & Van Tienderen, 2004) using 1000 bootstrap replicates to assess significance. To test for an isolation-by-distance pattern of genetic differentiation within the Lake Geneva drainage, the pairwise F_{ST} values were correlated with pairwise waterway distances using a Mantel test with 10,000 bootstrap replicates to assess significance. The genetic structure was further assessed using an admixture model implemented in STRUCTURE 2.3.4 (Falush *et al.*, 2007) with 30,000 burnin steps followed by 300,000 MCMC steps. The analysis was performed assuming any number of genetic clusters (K) between 1 and 15, with 10 replicates for each assumed K. The optimal number of clusters was determined based on the log likelihood of each run and its variation among runs for the same K, following Evanno et al. (2005). To further infer the genetic relationships among populations, a neighbour joining tree, based on Cavalli-Sforza distances among populations was inferred. Statistical support for each node of the inferred tree was obtained using a bootstrap procedure with 1000 replicates in PHYLIP 3.69 (Felsenstein 2011).

In addition, 639 individuals from all contemporary populations within the Rhone drainage were genotyped for *Stn382*, following the protocols of Peichel et al. (2004). This microsatellite flanks a 60 bp indel in intron 1 of the *Eda* gene, yielding either a 158 bp allele – Eda_L, associated with the low plated phenotype or a 218 bp allele – Eda_C, associated with the fully plated phenotype (Colosimo *et al.*, 2005). PCR products were analyzed on a 1.5 % agarose gel, and genotypes were scored by eye. *Stn382* was however excluded from the population based analyses above because this marker is potentially under strong selection (Barrett et al. 2008). To test for an association between the presence of the Eda_C allele and *habitat,* a generalized linear mixed model was used, treating population as a random factor and assuming a binomial distribution. Significance of differences between groups was assessed using a post hoc *z* tests.

Finally, assuming that the presence of the Eda_C allele in the Lake Geneva region coincides with the presence of other alleles from the Lake Constance region as has been found to be the case in other parts of Switzerland (Lucek *et al.*, 2010), the allele frequency of both types of markers were compared between habitats. Lake Constance specific alleles were defined as the private alleles of both Constance populations combined in comparison to the population from the Lake Geneva system that showed the smallest amount of introgression based on the STRUCTURE analysis, i.e. VDS6. The frequency of these Constance private alleles was subsequently estimated for all other populations in the Lake Geneva region. To further test for habitat-specific and potentially adaptive introgression of the Eda_C within the Lake Geneva system, the difference between the allele frequencies of both marker types was calculated for each population. Comparisons between habitats were performed using *t* tests. All statistical analyses were performed in R 2.15.1 (R Core Team 2012).

Table 2: *Pairwise P_{ST} and angle between the leading eigenvectors for each comparison of historical vs. contemporary samples within the same site are given for either all traits combined or subsets using either only defense or only feeding related traits. In addition, the relative ellipse size and eccentricity for both the historical and contemporary population samples are given with the respective percentage difference. See table 1 for details of each population. Significant P_{ST} values, i.e. where the 95% confidence interval exceeds zero, are highlighted in bold.*

Site	**Habitat**	**Year range**	**Traits**	P_{ST}	**95% CI**	**Angle**	p_{Angle}	**Relative ellipse size**			**Eccentricity**		
								Historical	Contemporary	Δ%	Historical	Contemporary	Δ%
VSS3	Stream	1921-2009	All	0.000	0.000-0.227	**55.9°**	**<0.001**	0.398	0.327	-17.8	2.660	1.369	-48.5
			Defense	0.000	0.000-0.041	**16.8°**	**<0.001**	0.246	0.099	-59.8	6.432	5.305	-17.5
			Feeding	**0.218**	0.120-0.340	**11.1°**	**0.016**	0.120	0.091	-24.2	3.110	8.321	+167.6
VDS6	Stream	1958-1964	All	**0.359**	0.138-0.506	**51.8°**	**<0.001**	0.569	0.690	+21.3	1.557	1.490	-4.3
			Defense	0.041	0.000-0.175	11.5°	0.158	0.248	0.221	-10.9	4.868	11.535	+137.0
			Feeding	0.000	0.000-0.128	6.3°	0.409	0.239	0.149	-37.7	4.333	5.651	+30.4
VDS6	Stream	1958-2012	All	**0.384**	0.119-0.519	**82.9°**	**<0.001**	0.527	0.426	-19.2	1.557	2.115	+35.8
			Defense	0.000	0.000-0.059	3.8°	0.714	0.249	0.173	-30.5	4.868	2.947	-39.5
			Feeding	0.075	0.000-0.288	28.0°	0.165	0.229	0.276	+20.5	4.333	1.790	-58.7
VDS6	Stream	1964-2012	All	0.000	0.000-0.110	**81.0°**	**<0.001**	0.781	0.460	-41.1	1.490	2.115	+42.0
			Defense	0.000	0.000-0.075	7.7°	0.297	0.220	0.171	-22.3	11.535	2.948	-74.4
			Feeding	0.000	0.000-0.122	24.6°	0.222	0.137	0.279	+103.6	5.651	1.790	-68.3
GES2	Stream	1979-2009	All	**0.326**	0.041-0.709	**10.9°**	**<0.001**	0.460	0.221	-52.0	1.664	1.870	+12.4
			Defense	**0.215**	0.071-0.390	9.2°	0.062	0.185	0.095	-48.6	2.680	4.022	+50.1
			Feeding	0.000	0.000-0.163	**4.4°**	**0.001**	0.180	0.082	-54.4	5.394	6.278	+16.4
VDL4	Lake	1967-2010	All	0.000	0.000-0.139	**41.7°**	**0.048**	0.519	0.271	-47.8	3.188	1.469	-53.9
			Defense	0.000	0.000-0.108	12.4°	0.189	0.437	0.102	-76.7	5.776	3.599	-37.7
			Feeding	0.004	0.000-0.226	5.9°	0.773	0.112	0.136	+21.4	6.208	1.453	-76.6
VDL6	Lake	1967-2012	All	**0.109**	0.013-0.251	**74.4°**	**<0.001**	0.421	0.422	+0.2	1.247	1.761	+41.2
			Defense	0.010	0.000-0.088	3.8°	0.486	0.187	0.110	-41.2	5.310	6.028	+13.4
			Feeding	0.016	0.000-0.120	7.7°	0.092	0.101	0.199	+97.0	5.479	5.057	-7.7

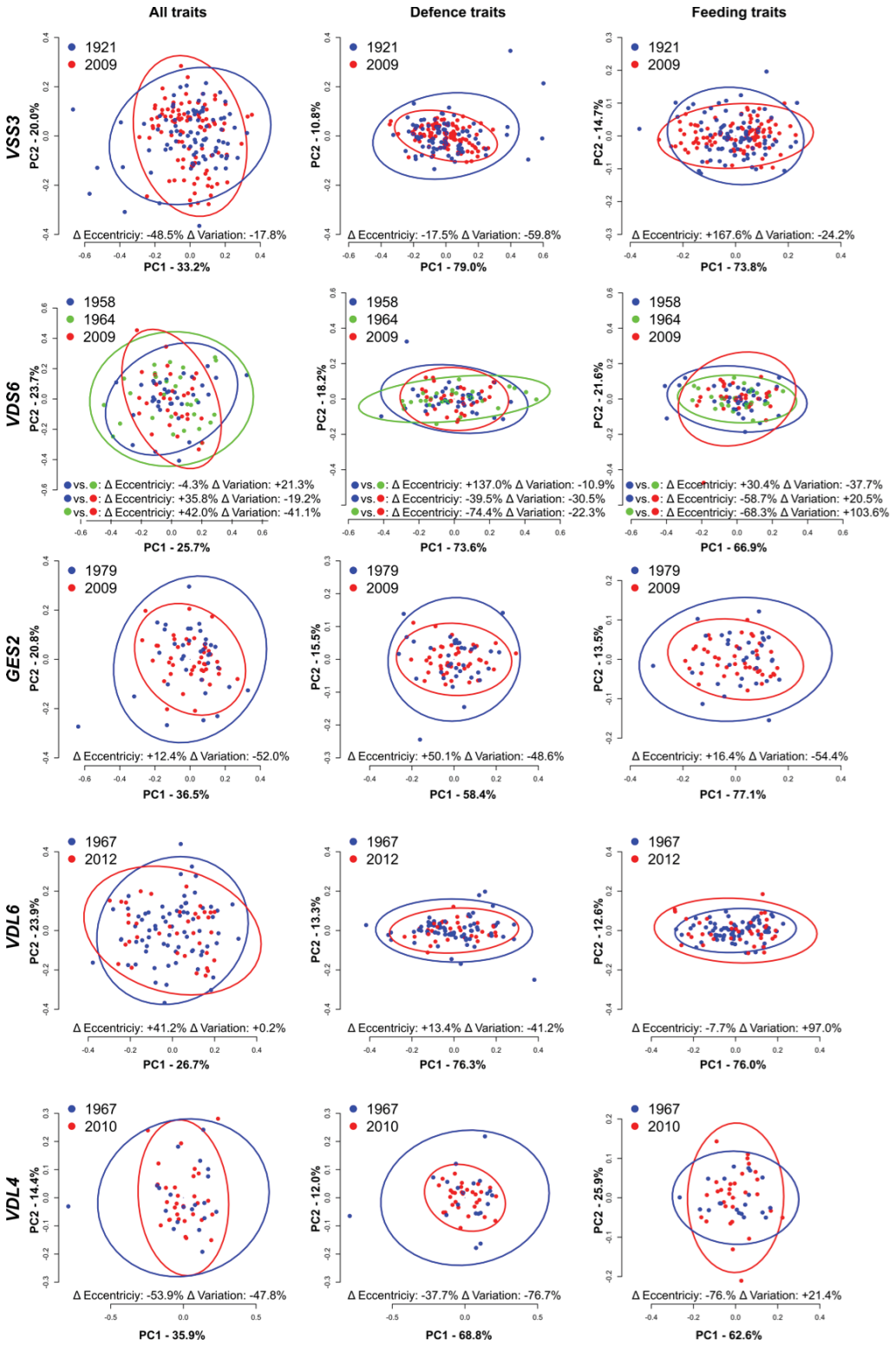

Figure 3: *Principal component scores based on either all linear morphological traits (left), defense related traits (middle) or feeding related traits only (right) for the two leading principal component axes. Shown are populations where both historical and contemporary samples were available. Ellipsoids represent the 95% CI with contemporary samples being colored in red and historical samples colored in blue and green. The relative changes over time in eccentricity and phenotypic variation, i.e. the size of the 95% CI ellipsoid, are indicated (see Table 2 for details).*

Results

Morphology

P matrices commonly changed both in their shape and their leading eigenvector $\boldsymbol{p}_{max}$ between historical and contemporary populations (Table 2, Figure 3). However, the evolutionary changes and the levels of pairwise phenotypic divergence (P_{ST}) varied among sites and trait combinations. P_{ST}s between historical and contemporary population samples from the same site were highly significant when all traits were combined (Table 2). Similarly when all traits were combined, $\boldsymbol{p}_{max}$ within sites differed significantly between historical and contemporary samples, i.e. showed a significant angle between historical and contemporary samples, in all cases. Using only feeding or defence related traits respectively, $\boldsymbol{p}_{max}$ did not statistically differ between historical and contemporary samples except for VSS3 for both trait categories and GES2, where for feeding related traits the angle between the historical and contemporary $\boldsymbol{p}_{max}$ was small but significant. The shape of the **P** matrix also changed over time (Figure 3), where the phenotypic variation captured by the two leading phenotypic axes decreased over time in many populations. This was true for all traits analysed together, and defence traits analysed separately (Table 2; Figure 3). Variation however increased in three of five populations for feeding related traits (VDS6, VDL6, VDL4). Concomitantly, eccentricity changed over time in many sites.

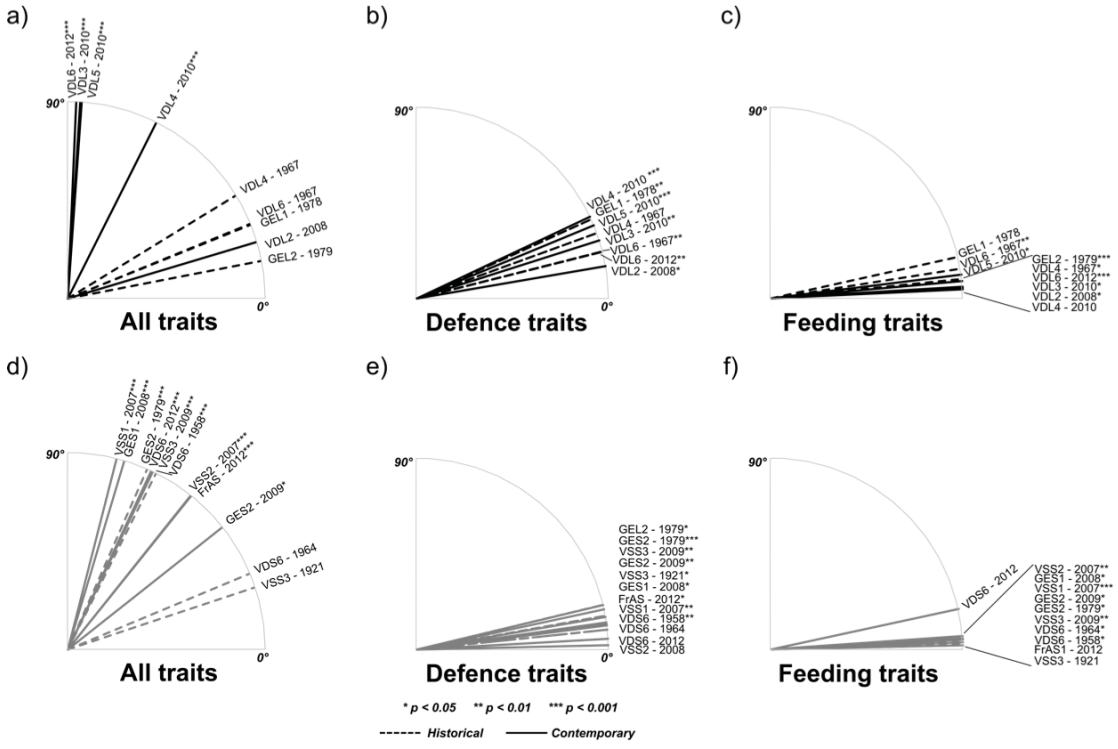

Figure 4: *Relative angle against a common* $\boldsymbol{p}_{max}$ *for each population and year for either all traits combined or defense or feeding related traits only (see Table S1). The relative axis (*$\boldsymbol{p}_{max}$*) for each population is given for lake (a - c) and stream (d - f) populations, separating historical (dashed) and contemporary (continuous line) sampling events. P values are based on a bootstrapping approach with 1000 replicates (see main text for details).*

When comparing all sampled populations, the best model explaining the angles between a local population's $\boldsymbol{p}_{max}$ and the common $\boldsymbol{p}_{max}$ when combining information from all phenotypic traits retained only *time* as significant factor: Historical populations show a significantly smaller angle against the common $\boldsymbol{p}_{max}$ than contemporary populations ($F_{1,18} = 10.4$, $p = 0.005$, Table 3, Figure 4a and d). When defence related traits were analysed on their own, the retained best model included only *habitat*, with lake populations having significantly larger angles against the common $\boldsymbol{p}_{max}$ ($F_{1,18} = 25.2$, $p < 0.001$, Figure 4b and e). Considering feeding related phenotypic traits on their own, the best statistical model showed a significant interaction between *habitat* and *time* ($F_{1,16} = 5.53$, $p = 0.032$), where historical lake populations had larger angles than contemporary ones against a common $\boldsymbol{p}_{max}$, whereas this difference was inversed for the stream populations (Figure 4c and f, Table 3). *Habitat* was furthermore marginally significant ($F_{1,16} = 4.53$, $p = 0.049$) with lake populations showing relatively larger angles than stream populations.

Table 3: *Angle between the leading eigenvector using all populations pooled and the leading eigenvector calculated for each population using either all linear measurements or a subset with defense or feeding related traits only. P values are based on bootstrapping with 1000 replicates (see main text for details). Significant values (p < 0.05) are highlighted in bold, values 0.10 < p < 0.05 are further highlighted in italics. See table 1 for details of each population.*

			All traits		***Defense traits***		***Feeding traits***	
Site	**Habitat**	**Year**	**Angle °**	***p***	**Angle °**	***p***	**Angle °**	***p***
VSS3	Stream	1921	*18.4*	*0.057*	**9.8**	**0.023**	1.2	0.139
VDS6	Stream	1958	**63.3**	**0.000**	**6.0**	**0.003**	**1.8**	**0.032**
VDS6	Stream	1964	22.7	0.192	6.0	0.107	**2.1**	**0.019**
GES2	Stream	1979	**66.3**	**0.000**	**13.5**	**0.000**	**2.2**	**0.033**
VSS1	Stream	2007	**75.7**	**0.000**	**7.3**	**0.001**	**3.0**	**0.000**
VSS2	Stream	2007	**51.4**	**0.000**	1.3	0.855	**4.0**	**0.002**
GES1	Stream	2008	**73.3**	**0.000**	**8.1**	**0.015**	**3.3**	**0.017**
GES2	Stream	2009	**38.4**	**0.027**	**10.2**	**0.005**	**2.9**	**0.013**
VSS3	Stream	2009	**65.0**	**0.000**	**12.1**	**0.002**	**2.1**	**0.002**
VDS6	Stream	2012	**64.5**	**0.000**	3.2	0.390	*12.2*	*0.086*
FrAS	Stream	2012	**51.2**	**0.000**	**7.6**	**0.018**	1.2	0.403
VDL4	Lake	1967	31.5	0.204	*20.0*	*0.060*	**5.4**	**0.027**
VDL6	Lake	1967	22.0	0.285	**14.2**	**0.007**	**8.9**	**0.002**
GEL1	Lake	1978	*22.3*	*0.075*	**24.4**	**0.004**	*12.5*	*0.094*
GEL2	Lake	1979	11.0	0.171	**14.1**	**0.027**	**5.8**	**0.000**
VDL2	Lake	2008	16.6	0.136	**9.7**	**0.030**	**3.2**	**0.042**
VDL3	Lake	2010	**86.0**	**0.000**	**17.7**	**0.001**	**3.5**	**0.015**
VDL4	Lake	2010	**63.4**	**0.000**	**25.3**	**0.000**	2.7	0.357
VDL5	Lake	2010	**87.6**	**0.000**	**22.5**	**0.000**	**7.1**	**0.011**
VDL6	Lake	2012	**86.4**	**0.000**	**13.5**	**0.001**	**5.2**	**0.001**
Average Stream (± 1 SD)			53.9 (± 20.6)		7.8 (± 3.8)		3.5 (± 3.2)	
Average Lake (± 1 SD)			47.4 (± 32.9)		17.9 (± 5.4)		6.0 (± 3.1)	

Genetic structure

The global F_{ST} values within the Lake Geneva system that were calculated separately for each marker did not statistically differ between putatively QTL linked and neutral markers (Wilcoxon W = 9, p = 0.999). Consequently all markers were pooled for the subsequent analyses. When analysing the contemporary samples from Lake Geneva together with those from France and the Swiss Rhine catchment, two genetic clusters (K=2) was the best supported K in STRUCTURE as inferred by the method of Evanno *et al.* (2005). The two clusters separate the Rhone drainage, including Lake Geneva, from populations in the Aare/Rhine drainage (Bern, Constance) except for Neuchatel (VDS2; Figure 5a), which was mainly assigned to the Rhone drainage cluster with clear indications of genetic admixture with the Aare/Rhine drainage stickleback. Introgression from the Aare/Rhine drainage was also observed, albeit to a lesser extent, among the populations in the Lake Geneva catchment, and in populations from the lake more so than in streams. In addition, similarly low levels of introgression from the Rhone drainage into the Rhine drainage were observed in the Bern populations. The neighbour joining tree further suggested a single genetic clade for all populations from the Rhone drainage, where the Neuchatel populations, together with the Bern populations are resolved as intermediate between Geneva and Constance, consistent with their hybrid origin (Figure 5b; Lucek et al. 2010).

The pairwise F_{ST} analysis supports genetic differentiation between the Lake Geneva populations and the populations from the Aare/Rhine drainage excluding Neuchatel (all F_{ST} >0.200 and p = 0.001; Table S1). The Neuchatel population (VDS2) showed significant genetic differentiation from all populations in the Lake Geneva region (all F_{ST} < 0.083 and p < 0.010), except for the geographically closest VDL2 lake population (F_{ST} = 0.008, p = 0.129). The population from the southern Rhone (FrAS) on the other hand showed substantial genetic differentiation from all populations in the Lake Geneva catchment (all F_{ST} ≥ 0.098 and p = 0.001). We observed considerable genetic differentiation within the Lake Geneva region (global F_{ST} = 0.030, p < 0.001), which was not significantly explained by geographic distance (Mantel r = 0.199, p = 0.069)**.** The overall genetic differentiation between habitats in the Lake Geneva region, pooling all individuals from streams and all from the lake, was even smaller (F_{ST} = 0.002, p =0.696), speaking against colonization of the two habitats by distinct stickleback lineages.

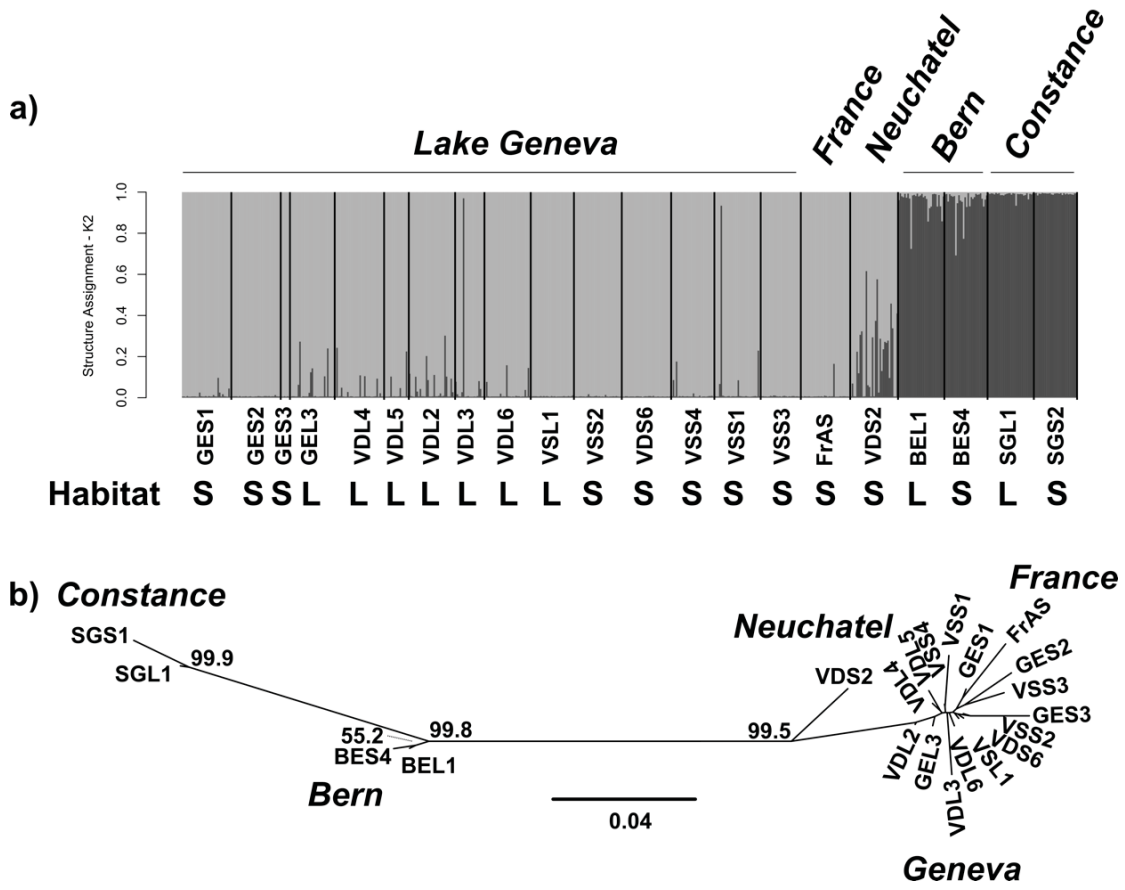

Figure 5: *Genetic relationship among individuals and populations within Lake Geneva and Switzerland (Constance, Bern and Neuchatel) as well as a population from France downstream Lake Geneva, using nine microsatellites. a) Individual based assignments using STRUCTURE for the best statistically supported number of clusters – K = 2. The respective habitat where populations were samples is indicated (L = Lake, S = Stream). b) Unrooted neighbour joining tree based on Cavalli-Sforza distances of population based allele frequencies. Bold numbers indicate the statistical support for each node based on 1000 bootstrap replicates. Only values with more than 50% bootstrap support are given. See table 1 for details of each population.*

Lateral plate phenotype/genotype-environment associations

In concordance with historical reports and consistent with the genetic lineage that dominates the lake, (Lucek *et al.*, 2010 and references therein), fully plated individuals were absent from Lake Geneva in all our historical population samples (Figure 6). Only exceptionally single individuals had few additional plates close to their structural plates, a phenotype that is not necessarily associated with the presence of the Eda_C allele (Lucek *et al.*, 2012b). In contrast, fully and intermediate plated individuals were numerous among our contemporary samples. The best linear mixed model to explain the overall variation in lateral plate number included a significant contribution of both *time* and *habitat* with a non significant interaction (*post hoc* $t_{19,1075} = 1.91$, $p = 0.072$). The number of lateral plates was significantly lower among historical samples

($t_{20,1075}$ = 3.01, p = 0.007) and lake dwelling individuals had significantly more plates than stream dwelling ones ($t_{20,1075}$ = 3.96, p = 0.001). Lake fish had significantly more plates when habitat dependent differentiation in lateral plate number was separately tested for historical and contemporary individuals (historical: $F_{1,6}$ = 10.8, p = 0.017; $F_{1,13}$ = 14.2, p = 0.002; Figure 6). Concomitantly with the occurrence of highly plated individuals among contemporary samples, the Eda_C allele occurs. The presence of the Eda_C allele was significantly higher in the lake than in the stream habitats for contemporary individuals (z = 4.24, p < 0.001), suggests habitat dependent selection and is consistent with the observed phenotypic differences in lateral plate numbers among contemporary populations. *Eda* was significantly correlated with lateral plate phenotypes (R^2 = 0.838, p < 0.001).

Within the Lake Geneva system, the frequency of Lake Constance private alleles did not statistically differ between habitats ($t_{1,13}$ = 1.84, p = 0.089; Figure 7). In contrast, the Eda_C allele frequency was significantly higher among lake populations ($t_{1,13}$ = 2.88, p = 0.027). The Eda_C allele frequency was significantly higher than expected from the frequency of other Constance derived alleles in the lake ($t_{1,13}$ = 2.82, p = 0.029).

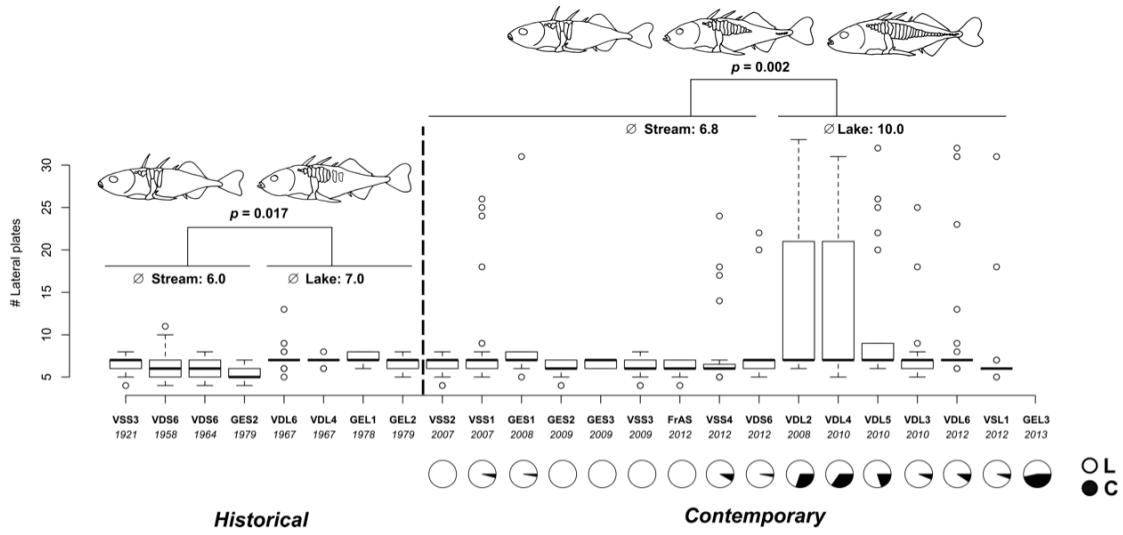

Figure 6: *Number of lateral plates per population for both historical and contemporary lake and stream populations. Significances between habitats are based on linear mixed effect models using population as a random factor. The average number of plates for each habitat is given. Pie plots indicate the allele frequency of the STN382 allele (white – L allele, black – C allele). No phenotypic data was available for the GEL3 population (see Table 1 for details).*

Discussion

Combining quantitative genetic and population genetic data, we explored the evolutionary trajectories that let to the previously described contemporary emergence of parapatric lake and stream stickleback ecotypes in Lake Geneva

(Lucek *et al.*, 2013). We had access to whole-preserved fish from museum collections starting in 1921, hence ~50 years after the arrival of stickleback, and had our own collections mostly from 2007 and 2008. Hence, we studied phenotypic evolution over the second half of the 140 years since colonization. Consistent with other studies on multivariate evolution in recently colonized habitats (Badyaev, 2010; Eroukhmanoff & Svensson, 2011; Calsbeek *et al.*, 2011), we find that the population based covariance (**P**) matrices changed over time in their shape, size and in some cases their orientation (Table 2, Figure 3). Overall, parapatric habitat dependent differentiation among ecotypes in feeding and defence-related traits seems to have evolved along similar evolutionary trajectories (Figure 4). This suggests a consistent divergent selective regime between the two habitats. Interestingly, lake and stream populations had consistently different **P** matrices already among our historical samples. This must therefore have evolved in the first ~50 years after the introduction and colonization of stickleback in Lake Geneva. For lateral plates, the extent of phenotypic differentiation between lake and stream stickleback has further increased from the historical to the contemporary samples (Figure 6). We find evidence for recent introgression of alleles from a distantly related lineage outside the Lake Geneva region that likely introduced a new allele at the *Eda* locus that is linked to highly plated phenotypes and which experiences itself habitat dependent selection (Figure 5 & 7). Thus introgression of adaptive variation through hybridisation seems to have promoted increased parapatric phenotypic divergence over the last decades (Rieseberg, 2011; Pardo-Diaz et al., 2012).

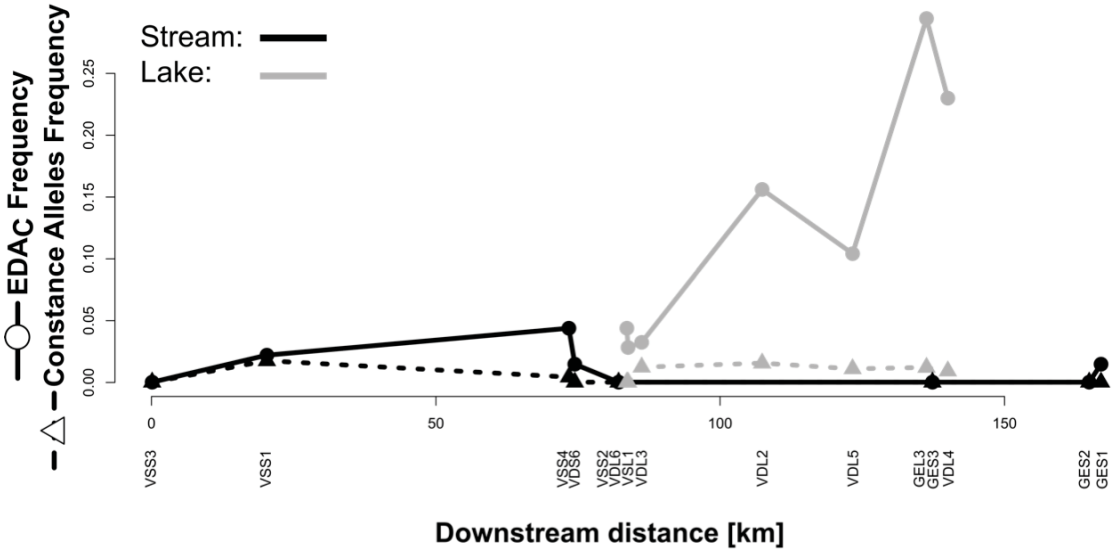

Figure 7: *Frequency of the Eda$_C$ allele (circles) and alleles deriving from the Lake Constance region (triangles) for each contemporary site within the Lake Geneva region. Sites are ordered according to the downstream distance from the VSS3 site. Stream sites are depicted in black, lake sites in grey.*

Evidence for consistent parapatric ecotypic differentiation

Both the occurrence and extent of parapatric ecotype formation depend on the underlying environmental and selective gradients (Endler, 1977; Doebeli & Dieckmann, 2003), as well as on the availability of similar genetic variation. Parallel evolutionary divergence is therefore only expected when the selective regimes are very similar among populations (Langerhans & DeWitt, 2004; Kaeuffer *et al.*, 2012) and adaptive genetic variation is not limiting (Barrett & Schluter, 2008). Cases of parapatric lake-stream stickleback systems provide both evidence for parallelism and nonparallelism in the realized trait-specific divergence that occur both on smaller geographical scales as well as between continents (Hendry & Taylor, 2004; Berner, 2009; Berner *et al.*, 2010; Kaeuffer *et al.*, 2012; Lucek *et al.*, 2013; Ravinet et al., 2013b).

Habitat dependent phenotypic divergence and ecotype formation along the lake-stream axis has been previously found for sticklebacks in the Lake Geneva system (Berner *et al.*, 2010; Lucek *et al.*, 2013), where ecotypes differ predominantly in defense and feeding related traits as well as body depth (Lucek *et al.*, 2013). Parallel habitat dependent selection for both feeding and defence related traits has been repeatedly found in stickleback and may reflect similar selection regimes (Reimchen *et al.*, 1985; Hendry *et al.*, 2002; Kaeuffer *et al.*, 2012; Lucek *et al.*, 2013; Ravinet *et al.*, 2013b): Whereas stream populations feed predominantly on benthic food and experience a macroinvertebrate dominated predation regime, lake populations may predominantly feed on zooplankton and are exposed to a predation regime dominated by piscivorous fish and birds (Reimchen, 1980; 1994; Hendry *et al.*, 2002; Berner *et al.*, 2008; Lucek *et al.*, 2012a; Ravinet *et al.*, 2013b). In line with this, we find that for defence and feeding related traits $\boldsymbol{p}_{\mathbf{max}}$ differs significantly and consistently between lake and stream habitats (Figure 4, Table 3), when comparing the leading eigenvectors $\boldsymbol{p}_{\mathbf{max}}$ of population based **P** matrices with the overall $\boldsymbol{p}_{\mathbf{max}}$ (*sensu* Schluter, 1996). For these traits, population based $\boldsymbol{p}_{\mathbf{max}}$ are moreover relatively similar to each other within a habitat type, suggesting that ecotype formation may proceed along consistent evolutionary trajectories through time and that the overall divergent selective regime was relatively stable through much of the time since colonization.

In contrast, population specific **P** matrices change generally between historical and contemporary samples of the same population even a hundred years after colonization (Figure 3, Table 2), where **P** matrices differ in terms of size, eccentricity and to some extent their directionality ($\boldsymbol{p}_{\mathbf{max}}$). Nevertheless two relatively common patterns emerge: First, phenotypic variation in defence related traits is lower among contemporary than among historical samples (Figure 3, Table 2). Secondly we find only few cases – none for defence related traits – where both P_{ST} and the angle between $\boldsymbol{p}_{\mathbf{max}}$ are significant between historical and contemporary samples, thus single populations seem to rarely evolve along $\boldsymbol{p}_{\mathbf{max}}$ over time. This suggests that adaptation to a local environment occurs relatively rapidly after its colonization especially for feeding and defence related trait, whereafter the average phenotype remains relatively stable over time and may experience stabilizing selection (Jones *et al.*, 2004).

The tempo and mode of ecotype formation

Adaptive phenotypic evolution can be rapid and emerge through phenotypic plasticity, selection on standing genetic variation or a combination of both (Robinson & Wilson, 1996; Thompson, 1998; Hairston *et al.*, 2005; Carroll *et al.*, 2007; Dlugosch & Parker, 2008). However, whereas phenotypic plasticity may instantaneously produce differentially adapted phenotypes (Ghalambor *et al.*, 2007), the evolution of genetically differentially adapted populations may occur gradually over time due to constraints to adaptation (Schluter, 1996). Consequently, the evolutionary trajectories that underlie divergent evolution should rapidly and consistently diverge if new adaptive phenotypes are achieved mainly by phenotypic plasticity (Lande, 2009; Draghi & Whitlock, 2012). Conversely, a gradual increase in divergence between distinctly adapted populations over many generations is expected if phenotypic evolution is mainly heritable, where the **G** matrix is realigning itself slowly towards a new adaptive peak on the adaptive landscape (Schluter, 1996; Steppan *et al.*, 2002). In the latter case, the accumulation of additional adaptive variation through introgression or hybridization may lead to a rapid realignment of the evolutionary trajectories (Guillaume & Whitlock, 2007).

We find that habitat dependent divergence in $\boldsymbol{p}_{\mathbf{max}}$ may evolve rapidly and may lead quickly to some phenotypic differentiation between the populations inhabiting these habitats as the evolutionary trajectories differed already consistently between habitats among our historical samples (Figure 4). However, we cannot tell from our data whether this early change was achieved by phenotypic plasticity or fast but gradual evolution because our data series only starts ~50 years post colonization. Phenotypic plasticity may in principle promote the colonization of distinct habitats and subsequent ecotype formation (Smith & Skúlason, 1996; Ghalambor *et al.*, 2007), by rapidly shifting the major phenotypic trajectory, where plastic trajectories are expected to align if populations experience similar selective regimes (Lande, 2009; Draghi & Whitlock, 2012). However, the fact that we still observe changes in the population specific **P** matrix even a hundred years after the colonization of the Lake Geneva system supports the alternative hypothesis of a gradual increase in differentiation over time and concordantly a heritable component of ecotypic divergence (Schluter, 1996). The relatively recent introgression from the strongly differentiated stickleback lineage dominating the East of Switzerland may have introduced additional adaptive genetic variation that facilitated the observed changes in the **P** matrix.

Evidence for adaptive introgression

Sticklebacks in the Lake Geneva region are thought to derive from a single introduction event about 140 years ago, originating from the Rhone River south of Lake Geneva (Fatio, 1882). This has been supported by mitochondrial haplotypes (Lucek *et al.*, 2010), which suggest that the originally introduced sticklebacks in the Lake Geneva region and the natural populations in the Rhone River belong to a genetic lineage that is fixed for both the low plated phenotype and the Eda_{L} allele (Munzing, 1963; Mäkinen & Merilä, 2008; Lucek *et al.*, 2010, *Lucek* unpublished data). Using nuclear markers, we find indications for a

secondary introduction and subsequent introgression from the Aare/Rhine system into the Lake Geneva system (Figure 5). Such secondary introductions may increase the potential for adaptive phenotypic divergence by increasing the genetic variation on which selection can act (Garant et al., 2007; Barrett & Schluter, 2008). This could be the case for the *Eda* gene in the Lake Geneva region, which underlies distinct lateral plate phenotypes (Colosimo et al., 2004; 2005). Both the different lateral plate phenotypes as well as the *Eda* gene itself are known to experience divergent habitat dependent selection (Reimchen, 1994; Barrett et al. 2008; Barrett, 2010; Zeller *et al.*, 2012a) and references therein), where even small changes in the average lateral plate number can be adaptive (Reimchen, 1994; 2000). A more fully plated body, and hence the presence of the Eda_C allele are thought to be beneficial to protect against attacks from piscivorous predators by increasing the propensity of surviving an attack (Reimchen, 1992; 1994). A lower number of plates and the associated Eda_L allele on the other hand, are thought to increase the rate of handling failures of macroinvertebrate predators (Reimchen, 1994; Marchinko, 2009).

We find that lake populations in the Lake Geneva region had a very slightly but significantly increased number of plates compared to stream populations already in the first half of the 20th century, prior to the inferred introgression event. Highly plated phenotypes and thus likely the Eda_C allele were absent (Figure 6). While historical individuals showed occasionally an increased number of lateral plates, these specific phenotypes can be expressed in the absence of the Eda_C allele (Colosimo *et al.*, 2005; Cano *et al.*, 2006; Le Rouzic *et al.*, 2011; Lucek *et al.*, 2012b). However, the phenotypic differentiation is much increased among contemporary populations, mainly due to the presence of many highly plated individuals in lake populations. Concomitantly we find the Eda_C allele to be present among our contemporary populations and in concordance with other studies to be significantly associated with the number of lateral plates (Colosimo *et al.*, 2005; Barrett *et al*., 2008). Given the observed introgression at microsatellite markers (Figure 5) it seems likely that the Eda_C allele was introduced by the same introgression event from the Aare/Rhine system. Indeed, Eda_C haplotypes in the Lake Geneva region are shared with the Aare/Rhine system (Berner *et al.*, 2010). Interestingly, the frequency of Eda_C exceeds that of other Lake Constance derived alleles within the lake environment significantly (Figure 7). This is consistent with adaptive introgression (Rieseberg, 2011; Pardo-Diaz *et al*., 2012), where positive selection leads to the disproportionate increase in the frequency of an adaptive allele. In our case, positive selection on Eda_C seems to be habitat dependent, acting only in lake populations.

Conclusions

Taken together, our results suggest a case of contemporary ecotype formation that is associated with consistent evolutionary divergence among populations through time and space. Moreover, parapatric ecotypes can evolve along similar phenotypic axes of divergence for ecologically relevant trait categories. Whereas divergent habitat dependent selection leads to some spatially consistent phenotypic differentiation among populations inhabiting these habitats early on, more recent adaptive introgression has facilitated further adaptive differentiation among ecotypes in anti-predator related traits.

Such processes may be common among invasive species and more generally during the colonization of new habitats.

Acknowledgement

We thank Sonia Fisch-Muller from the Muséum d'histoire naturelle de la Ville de Genève (NHMG) and Olivier Glaizot from the Musée Cantonal de Zoologie de Lausanne (NHML) for their support obtaining the historical samples. We are indebted to Mélissa Lemoine who provided valuable statistical support for the linear models and Fabrice Eroukhmanoff, Bänz Lundsgaard-Hansen, Joana Meier, David Marques for comments on earlier versions of the manuscript. This research was supported by Swiss National Science Foundation Grant 31003A-118293 to OS.

Supplement

Table S1: *Pairwise F_{ST} values for all genotyped contemporary populations based on nine microsatellites (lower triangle). Significant comparisons (p<0.05), based on 1000 bootstrap replicates are highlighted in bold, with the actual p values given in the upper triangle. See table 1 for details of each population.*

		Lake Geneva system															**Other populations**					
		GES1	GES2	GES3	GEL3	VDL4	VDL5	VDL2	VDL3	VDL6	VSL1	VSS2	VDS6	VSS4	VSS1	VSS3	FrAS1	VDS2	BEL1	BES4	SGL1	SGS1
Lake Geneva system	GES1	-	0.001	0.026	0.161	0.003	0.003	0.195	0.262	0.042	0.051	0.005	0.178	0.017	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001
	GES2	**0.079**	-	0.002	0.001	0.001	0.002	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	GES3	**0.056**	**0.214**	-	0.071	0.021	0.042	0.030	0.246	0.009	0.019	0.184	0.021	0.046	0.001	0.001	0.001	0.046	0.001	0.001	0.001	0.001
	GEL3	0.006	**0.068**	0.034	-	0.762	0.141	0.450	0.294	0.208	0.165	0.636	0.403	0.158	0.001	0.001	0.001	0.004	0.001	0.001	0.001	0.001
	VDL4	**0.027**	**0.069**	**0.057**	-0.006	-	0.246	0.027	0.011	0.083	0.061	0.751	0.019	0.115	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001
	VDL5	**0.043**	**0.067**	**0.068**	0.011	0.007	-	0.086	0.291	0.570	0.320	0.339	0.079	0.672	0.236	0.002	0.001	0.005	0.001	0.001	0.001	0.001
	VDL2	0.006	**0.049**	**0.048**	0.000	**0.017**	0.018	-	0.246	0.146	0.294	0.070	0.094	0.108	0.001	0.005	0.001	0.129	0.001	0.001	0.001	0.001
	VDL3	0.005	**0.075**	0.020	0.003	**0.024**	0.006	0.006	-	0.571	0.117	0.065	0.765	0.114	0.001	0.001	0.001	0.005	0.001	0.001	0.001	0.001
	VDL6	**0.015**	**0.041**	**0.071**	0.005	0.010	-0.004	0.007	-0.002	-	0.721	0.152	0.628	0.490	0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.001
	VSL1	0.015	**0.050**	**0.070**	0.007	0.013	0.004	0.003	0.012	-0.005	-	0.231	0.175	0.693	0.014	0.006	0.001	0.011	0.001	0.001	0.001	0.001
	VSS2	**0.024**	**0.092**	0.019	-0.004	-0.006	0.003	0.014	0.015	0.007	0.005	-	0.043	0.800	0.003	0.003	0.001	0.009	0.001	0.001	0.001	0.001
	VDS6	0.006	**0.065**	**0.063**	0.001	**0.021**	0.022	0.010	-0.008	-0.003	0.007	**0.017**	-	0.041	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	VSS4	**0.021**	**0.088**	**0.044**	0.007	0.009	-0.007	0.010	0.010	-0.001	-0.005	-0.007	**0.015**	-	0.143	0.007	0.001	0.010	0.001	0.001	0.001	0.001
	VSS1	**0.077**	**0.115**	**0.090**	**0.058**	**0.047**	0.007	**0.053**	**0.048**	**0.031**	**0.021**	**0.030**	**0.065**	0.008	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	VSS3	**0.057**	**0.102**	**0.112**	**0.046**	**0.043**	**0.052**	**0.030**	**0.072**	**0.049**	**0.035**	**0.032**	**0.072**	**0.029**	**0.050**	-	0.001	0.012	0.001	0.001	0.001	0.001
Other populations	FrAS1	**0.098**	**0.222**	**0.135**	**0.121**	**0.116**	**0.153**	**0.127**	**0.141**	**0.140**	**0.112**	**0.105**	**0.154**	**0.105**	**0.127**	**0.117**	-	0.001	0.001	0.001	0.001	0.001
	VDS2	**0.024**	**0.075**	**0.038**	**0.025**	**0.030**	**0.039**	0.008	**0.027**	**0.028**	**0.020**	**0.023**	**0.045**	**0.020**	**0.045**	**0.022**	**0.083**	-	0.001	0.001	0.001	0.001
	BEL1	**0.257**	**0.335**	**0.232**	**0.255**	**0.268**	**0.229**	**0.234**	**0.230**	**0.264**	**0.259**	**0.251**	**0.296**	**0.236**	**0.238**	**0.234**	**0.266**	**0.175**	-	0.584	0.001	0.001
	BES4	**0.262**	**0.337**	**0.246**	**0.259**	**0.272**	**0.237**	**0.240**	**0.236**	**0.274**	**0.272**	**0.262**	**0.302**	**0.251**	**0.254**	**0.242**	**0.269**	**0.185**	-0.002	-	0.001	0.001
	SGL1	**0.338**	**0.394**	**0.295**	**0.344**	**0.363**	**0.331**	**0.303**	**0.299**	**0.349**	**0.352**	**0.352**	**0.379**	**0.341**	**0.343**	**0.346**	**0.396**	**0.245**	**0.144**	**0.157**	-	0.040
	SGS1	**0.357**	**0.413**	**0.312**	**0.359**	**0.378**	**0.347**	**0.327**	**0.313**	**0.364**	**0.370**	**0.368**	**0.393**	**0.358**	**0.360**	**0.370**	**0.417**	**0.265**	**0.149**	**0.163**	**0.014**	-

Supplementary methods

Genomic DNA was extracted from fin tissue sample using a 10% Chelex solution, following the manufacturers protocol (Biorad, California, USA). 10 microsatellites were amplified in a single multiplexing set (Table S2).

The polymerase chain reaction (PCR) reactions consisted of 5 µl Qiagen Multiplexing Solution (Qiagen, Switzerland), 0.95 µl primer mix (Table S1), 3.05 µl dH_2O and 1 µl DNA per reaction. The PCR started with 15 min at 95°C followed by 26 cycles with 95°C for 30 seconds, 53°C for 90 seconds and 72°C for 60 seconds with a final elongation at 60°C for 30 minutes. PCR products were 1:10 diluted and visualized on a ABI 3130XL (Applied Biosystems, USA) following the manufacturers instruction. Alleles were scored using GENEMAPPER v4.0 (Applied Biosystems, USA).

Table S2: *Microsatellites used in this study with their fluorescent and concentration used. Primers and the position in the genome, i.e. linkage group, were obtained from Raeymaekers et al., 2007.*

Marker	QTL	Fluorescent	µl per reaction [10 µM]
Gaest66		Blue	0.1
STN30		Blue	0.1
STN96	2nd spine length	Blue	0.2
STN173		Green	0.05
STN196		Green	0.1
STN130	2nd spine length	Black	0.05
STN174		Black	0.1
STN185		Red	0.1
STN26	1st spine length	Red	0.05

Chapter 3

Repeated and predictable patterns of ecotypic differentiation during a biological invasion: lake-stream divergence in parapatric Swiss stickleback

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Abstract

The relative importance of ecological selection and geographical isolation in promoting and constraining genetic and phenotypic differentiation among populations is not always obvious. Interacting with divergent selection, restricted opportunity for gene flow may in some cases be as much a cause as a consequence of adaptation, with the latter being a hallmark of ecological speciation. Ecological speciation is well studied in parts of the native range of the threespined stickleback. Here we study this process in a recently invaded part of its range. Switzerland was colonized within the past 140 years from at least three different colonization events involving different stickleback lineages. They now occupy diverse habitats, ranging from small streams to the pelagic zone of large lakes. We use replicated systems of parapatric lake and stream populations, some of which trace their origins to different invasive lineages, to ask (i) whether phenotypic divergence occurred among populations inhabiting distinct habitats, (ii) whether trajectories of phenotypic divergence follow predictable parallel patterns, and (iii) whether gene flow constrains divergent adaptation or vice versa. We find consistent phenotypic divergence between populations occupying distinct habitats. This involves parallel evolution in several traits with known ecological relevance in independent evolutionary lineages. Adaptive divergence supersedes homogenizing gene flow even at a small spatial scale. We find evidence that adaptive phenotypic divergence places constraints on gene flow over and above that imposed by geographic distance, signaling the early onset of ecological speciation.

Introduction

The role of gene flow in either constraining or facilitating adaptive population divergence and speciation is a long-standing debate (e.g. Slatkin, 1987; Nosil & Crespi, 2004; Räsänen & Hendry, 2008; Abbott *et al.*, 2013). On the one hand, theory suggests that gene flow can impose important constraints on adaptive divergence by homogenizing allele frequencies and preventing the formation of co-adapted gene complexes (Haldane, 1948; Mayr, 1963; Slatkin, 1973; Endler, 1977; Slatkin, 1987; Hendry *et al.*, 2001). As a consequence, gene flow may hamper or completely prevent adaptive divergence and speciation. On the other hand, gene flow can also facilitate diversification by introducing adaptive genetic variation and increasing the adaptive potential of populations overall (Garant *et al.*, 2007; Abbott *et al.*, 2013). Migration can also be non-random with regards to environment and the resulting gene flow may thus also be adaptive (Edelaar & Bolnick, 2012), e.g. due to matching habitat choice, where individuals migrate to an environment that best matches their phenotype (Edelaar *et al.*, 2008; Bolnick *et al.*, 2009). When gene flow is maladaptive, adaptive divergence can impose itself a constraint on gene flow, namely when divergent natural and/or sexual selection cause extrinsic reproductive isolation (Schluter, 2000; Maan & Seehausen, 2011). Understanding the relationship and the balance between adaptive divergence and gene flow is therefore essential understanding the relative importance of selection and geographical isolation during speciation (Mayr, 1963; Endler, 1977; Hendry *et al.*, 2001; Nosil & Crespi, 2004; Coyne & Orr, 2004; Räsänen & Hendry, 2008). Doing so, however, requires studying the very early stages of replicated ecotypic divergence before strong extrinsic (and any intrinsic) reproductive isolation has evolved (Hendry *et al.*, 2000; Shafer & Wolf, 2013).

Adaptive population divergence may be repeated and predictable if the underlying divergent selection regime is comparable, similar genetic variation is present, and if maladaptive gene flow is not too strong (Endler, 1977; Doebeli & Dieckmann, 2003; Räsänen & Hendry, 2008). Indeed, ecological adaptation leads to parallel phenotypic differentiation in ecologically relevant traits in population pairs occupying different ecological contrasts (Schluter, 2000), where selection reduces phenotypic overlap coupled with adaptation to different adaptive peaks (Leimar *et al.*, 2008). Such phenotypic adaptation can occur despite gene flow if selection is sufficiently strong or migration is non-random with regard to adaptation as in habitat matching (Edelaar & Bolnick, 2012). Phenotypic divergence of populations can be initiated by ecological specialization and phenotypic plasticity at the individual level (Pfennig *et al.*, 2010), and can itself precede the origin of measurable reproductive isolation.

Ecological speciation in parapatry is often associated with adaptation to different environments and occurs often along environmental gradients (Endler, 1977; Dieckmann *et al.*, 2004; Terai *et al.*, 2006; Ingram, 2011). This has been studied in parapatric threespined stickleback (*Gasterosteus aculeatus*) lake-stream systems, which mostly evolved after the last glacial maximum (Hagen & Gilbertson, 1972; Gross & Anderson, 1984; Reimchen *et al.*, 1985; Hendry & Taylor, 2004; Berner *et al.*, 2008; 2009; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b; but see Berner *et al.*, 2010; Hendry *et al.*, 2013). Stream populations in

these systems often exhibit morphological features more conducive to feeding on benthic river invertebrates, while lake populations fall along a continuum between two possible ecotypes, one feeding on benthic invertebrates associated with the littoral zone and the other feeding on plankton in the limnetic zone of lakes. Although the divergence of stickleback ecotypes has in some instances occurred despite a high potential for gene flow (Schluter & McPhail, 1992; Rundle *et al.*, 2000; Hendry *et al.*, 2001; Berner *et al.*, 2009; Roesti *et al.*, 2012), in others, divergence seems constrained by gene flow due to potential genetic constraints (Hendry *et al.*, 2002; Berner *et al.*, 2010) or the time since divergence (Berner *et al.*, 2010; Hendry *et al.*, 2013).

Most evidence for the role of divergent environments in promoting adaptive divergence and ecological speciation, however, comes from long established populations, where the processes that underlie adaptive divergence are difficult to infer. In particular, ecological speciation has been studied in evolutionarily young systems, such as cichlid fishes in Nicaraguan lakes (Elmer *et al.*, 2010) and Lake Victoria (Seehausen et al., 2008a) or cases of postglacial colonization and diversification of freshwater fishes in north temperate lakes (e.g. Sandlund, et al., 1992a; Schluter, 2000; Bernatchez *et al.*, 2010; Hudson *et al.*, 2011) and parapatric lake-stream systems in stickleback (Hagen & Gilbertson, 1972; Reimchen *et al.*, 1985; Hendry & Taylor, 2004; Berner *et al.*, 2008; 2009; Kaeuffer *et al.*, 2012). Accrued empirical evidence suggests that ecological divergence can sometimes occur rapidly over just a few generations (e.g. Hendry *et al.*, 2000; Eroukhmanoff *et al.*, 2009; Leaver & Reimchen, 2012; see Hendry *et al.*, 2007 for a review). Rapid ecological divergence has also been shown during biological invasions (Hendry *et al.*, 2000; Phillips & Shine, 2006; Westley, 2011), which do provide great opportunities to study the very early stages of adaptive divergence and, in some cases, ecological speciation (Prentis *et al.*, 2008; Yoder *et al.*, 2010; Westley, 2011). Consequently, studying successful invasions with range expansion into several distinct habitat niches associated with phenotypic divergence may help clarify the ecological and genetic constraints that need to be surmounted during the early stages of ecological speciation.

In Switzerland stickleback were restricted to tributaries of the Rhine near Basel north of the Jura mountains, being absent from the Swiss midlands until about 1870 (Heller, 1870; Fatio, 1882; Bertin, 1925; Lucek *et al.*, 2010). Following subsequent introductions and the channelization of many Swiss waterways for irrigation (Heller, 1870; Fatio, 1882; Bertin, 1925), stickleback underwent a range expansion and now occur in large parts of the country, occupying a wide range of different habitats, ranging from tiny streams to very large lakes with vast pelagic zones (Lucek *et al.*, 2010). Consequently they provide an exceptional opportunity to study the replicated parallel initiation of ecotypic differentiation over short evolutionary timescales (~140 generations, Table 1). These historically independent and replicated lake-stream habitat contrasts also provide opportunities to examine the relationship between gene flow and divergence under variable levels of geographical isolation. Mitochondrial DNA surveys from populations across the country revealed the colonization of Switzerland by three distant lineages from different parts of Europe (Lucek *et al.*, 2010). The Lake Constance area (Fig. 1) is dominated by East European haplotypes from the Baltic region, whereas the Lake Geneva area

is dominated by a lineage typical of the lower Rhone river valley from the Mediterranean drainage. A third presumably native Swiss lineage dominates the Basel region (Lucek *et al.*, 2010). From these presumed native and introduction sites, the three lineages expanded into the Swiss midlands and met in large parts of northern and western Switzerland. In places like Lakes Neuchatel, Biel, and Wohlen (Bern; Fig. 1), populations have a mix of all mitochondrial haplotypes associated with a considerable elevation in haplotypic richness. Nuclear markers (AFLPs) also suggest admixture between the major lineages in these areas, and stickleback from here also display a marked increase in phenotypic diversity and variation (Lucek *et al.*, 2010). The midlands of Switzerland are characterized by many large and deep lakes, some oligotrophic, others meso- and eutrophic, lying in a rich network of streams and canals, which overall leads to extreme habitat contrasts between streams and lakes.

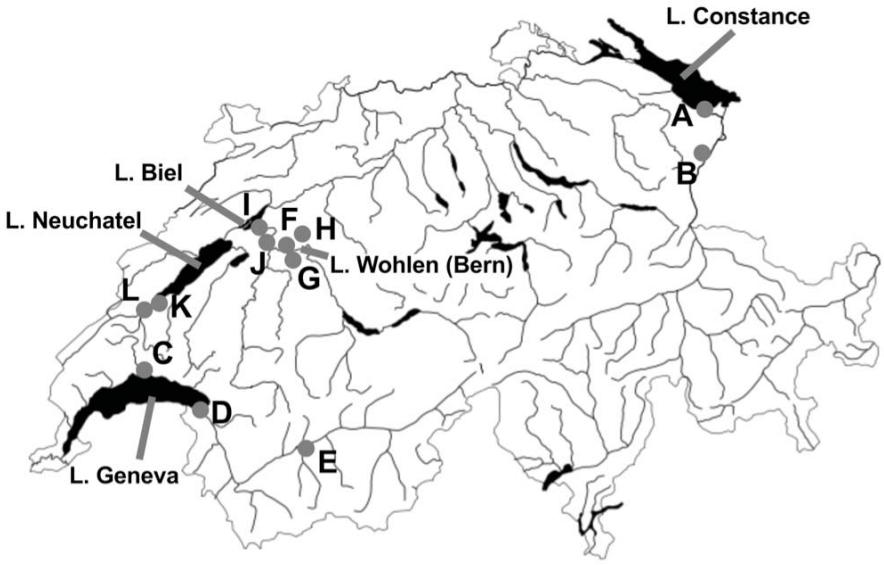

Figure 1*: Stickleback sampling sites used in this study: A – Constance L; B – Constance S; C – Geneva L; D – Geneva M; E – Geneva S; F – Bern L; G – Bern M; H – Bern S; I – Biel L; J – Biel S; K – Neuchatel S1 (near lake); L – Neuchatel S2 (upstream). Sample sites belong to the Rhine drainage (A, B); Rhone drainage (C-E), Aare drainage (F-J), or the Orbe drainage (K, L) respectively (See Table 1 for details; © Wikimedia).*

Here, we ask whether the recent range expansion of threespined stickleback in Switzerland is repeatedly and predictably associated with the onset of ecotypic differentiation between the major habitats, and to what extent the associated divergence in phenotypes is predictable. We contrast populations inhabiting three large, deep and oligo- to mesotrophic lakes and their associated streams, and one much smaller, shallower eutrophic lake and its associated streams. Specifically, we assess whether appreciable trait divergence has occurred over this short timescale, whether it is repeatable, and whether it is measurably constrained by the opportunity for gene flow. Finally we evaluate whether adaptive divergence constrains gene flow, i.e. whether we can detect signals of the early onset of ecological speciation (Nosil *et al.*, 2009). To address

these questions, we investigate variation and divergence at genetic markers, putatively functional phenotypic traits (armour, linear morphology of head and jaws, morphometric shape) and resource use inferred from stable isotopes.

Table 1: *Characteristics of sampling sites for Swiss sticklebacks used in this study with coordinates and sample sizes used for microsatellite and geometric morphometrics (N). The expected heterozygosity (H_E) is based on 17 microsatellites is furthermore indicated. Abbreviations for habitats: L, lake; S, stream; M, mouth of stream near its inflow into the lake. Introduction dates based on historical reports (Lucek et al., 2010) refer to lake systems, rather than to specific sites or habitats. The age of Lake Wohlen, a man-made dam is indicated too.*

System	Habitat	N	E	Waterway distance to lake [km]	Altitude above lake [m]	N	Introduction	H_E
Constance	L	47°29'08"	9°32'37"	<0.1	-	30	~1870*	0.551
	S	47°19'33"	9°34'41"	27.1	23	50		0.511
Geneva	L	46°31'02"	6°34'41"	0.0	-	38	~1870 †,‡	0.485
	M	46°23'07"	6°51'30"	<0.2	3	60		0.490
	S	46°12'50"	7°18'53"	61.0	92	35		0.470
Biel	L	47°54'57"	7°11'59"	<0.1	-	27		0.614
	S	46°58'58"	7°15'07"	16.5	33	36		0.625
Bern	L	46°57'59"	7°21'08"	0.0	-	33	After 1921§	0.623
	M	46°57'41"	7°22'46"	0.3	1	34		0.605
	S	46°59'30"	7°24'42"	14.6	90	28		0.610
Neuchatel	S1	46°47'31"	6°37'43"	0.3	4	35	~1920‡	0.492
	S2	46°38'30"	6°37'36"	1.1	1	31		0.524

* (Heller, 1870); † (Fatio, 1882); ‡ (Bertin, 1925); § Construction date of the Lake Wohlen dam

Methods

Sampling sites

We sampled stickleback populations inhabiting ecologically contrasting habitats potentially connected by gene flow in five lake systems of Switzerland: three large natural lakes and associated streams (systems of Lakes Constance, Geneva, and Biel), one smaller man-made lake (Lake Wohlen, Bern) and its associated streams, and two streams associated with Lake Neuchatel, (Fig. 1; Table 1). In the case of Neuchatel, no lake dwelling populations could be obtained during our screening of the area. Population abbreviations indicate the name of the lake system from which they were obtained followed by a habitat dependent indicator (L – lake, S – stream, M – stream mouth). We collected the lake dwelling sticklebacks on their breeding grounds (i.e., canals adjacent to the lake shore or small stream inlets as well as marinas within the lakes) to obtain adult phenotypes and because the large, deep and oligotrophic Swiss lakes make collecting stickleback in the pelagic where they feed during fall and winter nearly impossible. Here we classified breeding populations in lake inlets as lake

populations when the presence of adults was restricted to the breeding season (e.g., Constance L; Biel L), and as stream resident populations when adults were present year round (e.g., Geneva M; Bern S). Such information was unavailable for the Neuchatel system and as a consequence, we refer to these two collections as stream samples with different distances from the lake (near-lake and upstream). In the Geneva and Bern systems, we sampled three sites; a lake site, a stream site very near its outflow to the lake (stream-mouth), and an upstream site. Using hand nets and minnow traps, we collected sticklebacks between April and August 2007 and 2008. Sample sizes varied from 27 to 60 individuals per location (Table 1). We photographed each fish alive in the field in a standardized photo cuvette (10 x 10 x 2.5 cm). To avoid parallax error, we confined the fish to a space barely wider than its body and preventing its movements temporarily using a plastic panel. Fish were then sacrificed with an overdose of anaesthetic MS-222 and preserved in individual tubes with 95% ethanol.

Genetic differentiation

We extracted genomic DNA from fin tissue and genotyped eighteen microsatellite loci, selected from Peichel *et al.* (2001) and located on 15 of 26 linkage groups. Seven of these markers have been shown to be associated with known QTLs for spine lengths, the number of lateral plates and gill rakers (Peichel *et al.*, 2001). For these markers, we predict that, if they are linked to a phenotype under divergent selection, habitat dependent divergent selection should lead to an increased parapatric genetic differentiation relative to that in neutral markers. A detailed description of each marker together with the PCR and multiplexing protocols are available in the supplementary methods.

To evaluate genetic diversity observed in Swiss populations relative to that observed throughout the European range, we compared expected heterozygosities in Swiss samples to those reported from 58 populations sampled from the entire spectrum of other European freshwater habitats similarly genotyped at 18 microsatellite loci (Mäkinen *et al.*, 2006). We measured the pairwise genetic distance between collected samples as F_{ST} and assessed their *P*-values from 10'000 permutations (Meirmans & Van Tienderen, 2004). To quantify the relative importance of lake system versus habitat nested in lake system in the partitioning of genetic variation we employed an analysis of molecular variance (AMOVA) using GENODIVE 2.0 (Meirmans & Van Tienderen, 2004). In addition, we generated a genetic tree-like relationship among populations based on their pairwise F_{ST}s using 1000 bootstrapped resampling replicates to assess significance based on a neighbour-joining algorithm implemented in the program PHYLIP 3.69 (Felsenstein 2012). Finally we assessed genetic clustering within each lake-stream system, excluding Neuchatel using STRUCTURE 2.3.3 (Falush *et al.*, 2007) based on an admixture model implemented in with 30'000 burnin steps followed by 300'000 MCMC steps. For each system, we took the sampling location as prior information for the clustering due to the low expected level of genetic differentiation given the evolutionary age of the systems (Hubisz *et al.*, 2009).

Phenotypic measurements

We measured sixteen linear traits that are related to feeding ecology, anti-predator defence or general body shape and swimming behavior (Kristjánsson *et al.*, 2002a; Mori & Takamura, 2004; Berner *et al.*, 2008; Hendry *et al.*, 2011) and references therein) on each individual to the nearest 0.01 mm using a digital caliper (see Fig. 2 for details). We also counted the total number of gill rakers for each individual and took the mean length of the 2nd to 4th rakers, as counted from the joint of the dorsal arch bone, on the first lower gill arch using a micrometer mounted to a dissection microscope following Berner *et al.* (2008).

Since all linear measurements were significantly correlated with standard length (results not shown), we regressed each trait against standard length over all individuals, retaining the residuals. By pooling all systems, allometric information in some populations may be retained if the allometric trajectories differ between populations from different study systems. This allows however to estimate the system specific component of phenotypic variation, which is explained by different historical contingencies. Because all individuals are treated the same, the estimates of habitat-dependent parapatric differentiation should reflect the actual degree of divergence. All further analyses based on linear measurements are consequently based on these overall scale-free residuals. We analysed traits either separately or combined using principal component analyses (PCA) based on covariance matrices. PCAs combined either all linear traits or only traits that are linked to anti-predator defence (FSL, SSL, PSL, PGL; Fig. 2) or feeding (HL, ED, SnL, UJL, SnW, GRL). Especially the number of gill rakers (GRN) and their length (GRL), have been shown to be related to diet in stickleback (Bentzen & McPhail, 1984; Schluter & McPhail, 1992; Robinson, 2000) and other fish species (Gibson, 1988; MacNeill & Brandt, 1990; Lundsgaard-Hansen *et al.*, 2013).

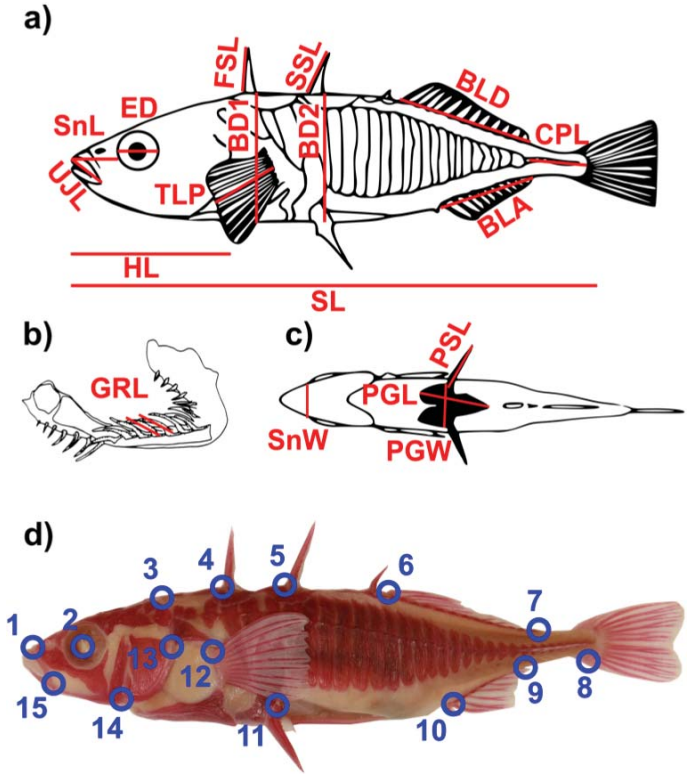

Figure 2: *Summary of all morphological measurements used in this study for linear measurements a)-c), which were either obtained on the left side a), the gill arch b) or from the ventral side of each individual as well as geometric morphometric landmarks d). An Alizarin red stained individual is shown to highlight the geometric morphometric landmarks used in this study. Linear measurements were as follow: FSL - length of the 1st dorsal spine, SSL - length of the 2nd dorsal spine, PSL - length of the pelvic spine, PGL - length of the pelvic girdle, HL - head length, UJL - upper jaw length, SnL - snout length, SnW - snout width, ED - eye diameter, SL - standard length, PGW - width of the pelvic girdle, BD1 -body depth measured after the 1st dorsal spine, BD2 - body depth measured after the 2nd dorsal spine, CPL - caudal peduncle length, BLA - basal length of the anal fin, BLD - basal length of the dorsal fin, TLP - total length of the pelvic fin. In addition, the length of the 3rd and 4th gill raker were measured. Geometric morphometric landmarks were as follow: 1 - anterior-most point of premaxillary bone, 2 - centre of the eye, 3 - junction of head and dorsal scales. 4 - insertion of the 1st spine, 5 - insertion of the 2nd spine, 6 - anterior end of dorsal fin, 7 - posterior end of dorsal fin, 8 - junction of lower caudal peduncle and tail fin, 9 - posterior end of anal fin, 10 - anterior end of anal fin, 11 - posterior junction of pelvic spine and body, 12 - upper insertion point of pectoral fin, 13 - posterior edge of operculum, 14 - ventral inflexion of preopercular bone, 15 - posterior-most point of premaxillary bone.*

In addition, we measured the overall morphometric shape using fifteen landmarks that were placed on standardised photographs using the software tpsDIG2 (Rohlf 2006; Fig. 2) and then used MorphoJ (Klingenberg, 2011) to analyse the landmark coordinates. Here, we first regressed partial warp scores against standard length of the fish to correct for allometry, followed by a PCA based on a covariance matrix using Procrustes distances of the regression residuals. Because allometric effects of body size may be retained, we subsequently tested each PC axis for a statistical association with standard length using linear models.

Parallelism and nonparallelism of phenotypic differentiation

To estimate the relative degree of phenotypic differentiation among populations, we estimated P_{ST}, an analog to Q_{ST} (Spitze, 1993) based on phenotypic measurements from wild individuals, using the approach of Kaeuffer et al. (Kaeuffer *et al.*, 2012). We use P_{ST} as a unitless and scale-free proportional measurement of pairwise difference and also to infer divergent selection on a trait by comparison with neutral genetic marker F_{ST} (Merilä & Crnokrak, 2001). As pointed out by several authors (Hendry, 2002; Edelaar & Björklund, 2011), P_{ST} should only be used for the latter in evolutionarily young and closely related populations assuming similar intra-population variation and mutation rates. With these caveats in mind, we nevertheless compare P_{ST} values with their respective F_{ST} to infer divergent selection only between parapatric populations. We calculated pairwise P_{ST} between populations using each linear trait and the number of gill rakers separately, and based on the scores of the first PC for all linear traits combined or separated into feeding or defense traits. This was also

done using the scores of the first PC based on morphometric shape. For each P_{ST} value, we estimated the 95% confidence interval using a resampling approach with 1000 replicates. To further assess the directionality of the parapatric phenotypic divergence, we performed pairwise *t*-tests using the number of gill rakers as well as size corrected trait values for linear measurements (statistics not shown). Because different landmarks were used among the different studies to assess morphometric body shape, the trait loadings of each PC analysis may differ. Consequently we did not assess directionality for morphometric body shape.

In order to estimate the relative contributions of *habitat* (lake or stream), *system* (Bern, Biel, Constance, Geneva), and their interaction on divergence between lake and stream stickleback, we estimated the percentage of non-error variance explained by each factor and their interaction based on their respective sums of squares using a sequential ANOVA model (Langerhans & DeWitt, 2004; Eroukhmanoff *et al.*, 2009). Here, the *habitat* term ought to reflect parallel parapatric divergent adaptation. The *system* term should reflect variation explained between parapatric lake-stream systems and thus likely reflect historical contingencies or environmental differences between lake-stream systems. Finally, the *system* x *habitat* interaction should account for the combined effects of system related historical contingency and ecotypic differentiation (Langerhans & DeWitt, 2004; Eroukhmanoff *et al.*, 2009). We calculated these estimates for all linear traits, the number of gill rakers, the scores of the first PC for all linear traits combined or separated into feeding or defense traits, and the scores of the first PC based on morphometric shape.

Testing for ecological speciation

A core prediction of ecological speciation theory is that adaptive phenotypic divergence between populations suppresses gene flow beyond what is explained by geographical distance, i.e. isolation by adaptation (Nosil *et al.*, 2009; Shafer & Wolf, 2013). To test this, we used P_{ST} and the geographical distance between parapatric populations to predict F_{ST} either on their own or combined. Because the strength of divergent selection may differ among traits and functional trait categories, we estimated P_{ST} for each trait as well as for the leading PC axis combining all traits, defence related traits, feeding related traits and shape. We measured the pairwise geographic distance as the minimal waterway distance between sampling sites (estimated in GOOGLE EARTH 6.1, Google, CA, USA). Because the stream gradient between parapatric sites may be a better predictor for the potential of gene flow than geographical distance (Caldera & Bolnick, 2008), we additionally performed all analyses using the altitudinal difference between sites instead of geographical distance (Table 1). Divergence values from all parapatric comparisons were included in these models (N = 9). Because we had three different population contrasts (two stream populations and one lake population) in the Bern and Geneva lake-stream systems, and to account for potential effects of pseudo-replication, we also calculated the same linear models using each only one out of three population contrasts from these systems. This results in nine different possible

combinations comprising five parapatric comparisons each. We then compared the R^2 values from these reduced models to the observed R^2 value of the model using all parapatric comparisons with a one-sample *t*-test. If the resampled R^2 values do not differ from the observed value, the repeated use of some populations in different population contrasts within the same system should not affect the overall conclusion.

Comparative parapatric differentiation

A powerful way to infer the pervasiveness of habitat-dependent parallel divergence among Swiss stickleback is to compare the Swiss systems with other parapatric lake-stream systems elsewhere in the world. For this we used published data from comparable systems in Canada (Kaeuffer *et al.*, 2012) and Ireland (Ravinet *et al.,* 2013b). We also added published data from two Swiss systems, comprising additional parapatric contrasts from Lake Geneva and Constance (Berner *et al.*, 2010). We obtained the parapatric F_{ST} estimates for these population contrasts from the summary tables in the respective publications and the original morphological data from the Dryad Digital Repository (doi: 10.5061/dryad.1960, 10.5061/dryad.k987h, 10.1111/jeb.12049). In all cases, we applied the same size correction as to the Swiss populations studied here (see above) except to the number of gill rakers, which we did not transform. We then estimated phenotypic differentiation based on P_{ST} for morphometric shape, the length and number of gill rakers, the length of the first and second dorsal spine as well as the length of the pelvic spine. All statistical analyses were performed in R 2.14 (R core development team 2012).

Stable isotopes

To test for differences in resource use among individuals inhabiting contrasting environments within lake systems, we used a subset of ten individuals from each population from all lake-stream systems (i.e. excluding the two Neuchatel stream sites) for stable isotope analyses of nitrogen (^{15}N) and carbon (^{13}C). To establish baseline SI signatures for ^{15}N and ^{13}C, we collected primary consumers for each site, sampling benthic invertebrates for streams and pelagic zooplankton for lakes at or close to the sampling site, depending of whether lake fish were sampled in the lake or in a nearby stream. Baseline samples were collected syntopically with the fish and during the same time of year under the same standardized conditions. We collected pelagic zooplankton from each lake over three 15-minute plankton tows with a 170 µm net. We then concentrated the zooplankton and stored it in 95% ethanol. Although not filtered to remove predatory species, because all pelagic zooplankton samples were treated similarly, errors introduced in baseline values from unwanted species were likely small and applied evenly to all samples. In streams, we collected 5-10 gastropods (Lymnaeidae) and stored them in 95% ethanol. We prepared the fish tissue as described by Paterson *et al.* (Paterson *et al.*, 2006), modified to also incorporate baseline samples. Briefly, we excised a 1.5 x 0.8 cm piece of muscle tissue from the right flank of each fish specimen. For each lake, we pooled

zooplankton samples into a single sample and used them as whole body homogenates; we did the same with the soft body of gastropods after their shells were removed. We subsequently dried all samples in an oven at 75˚C for 48 hours. We then placed the dried samples in clean solvent rinsed glass mortar and pestle and pulverized them into a homogenous powder. For sample, we placed 0.25 - 0.28 mg of the powder in a tin capsule (3.2mm; Elemental Microanalysis, Okehampton, UK), folded it into a small cube and placed it into a standard 96 well sample plate. Samples were processed at the Environmental Isotope Laboratory (University of Waterloo, ON, Canada) using a Micromass Isochrom-EA continuous flow stable isotope ratio mass spectrometer. Resulting SI ratios for each sample were given as deviations from standard reference materials (Pee Bee belemnite limestone for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$). For quality control and assurance, laboratory standards (uwEILAB, Waterloo, ON, Canada) were analyzed every five samples and we included 13% of all samples as duplicates (including all baseline samples).

In order to compare the trophic position among populations within systems, we corrected the obtained $\delta^{15}N$ values using population-specific baseline values following Post (2002). Trophic position differences greater than 1 typically indicate substantially different trophic levels among populations assuming a trophic enrichment of 3.4 ‰ for $\delta^{15}N$ (Post, 2002). Once converted to trophic position, we tested whether or not absolute mean differences in trophic positions among parapatric populations was significantly less than 1 using 10 000 Monte Carlo randomizations of individuals within each population.

For the $\delta^{13}C$ values, we applied a simple 2-source mixing model as demonstrated by McCutchan et al. (2003) to generate a proportion of pelagic and benthic/littoral carbon sources for each individual within systems. Here, we used the pelagic and benthic/littoral baseline $\delta^{13}C$ from each system as the two input sources, applying a 1.3 ‰ trophic enrichment factor (see McCutchan *et al.*, 2003). Because of the nature of the 2-source mixing model, especially when applying a trophic enrichment factor, it is not abnormal for carbon source proportions to sometimes be > 1.0 or < 0.0. This is an inherent problem of simple 2-source mixing models, which likely over-simplify or incompletely characterize carbon sources within such complex systems. However, the application of more complex models would require $\delta^{13}C$ values of more sources. With these caveats in mind, we nevertheless used this model to gauge the relative carbon sources among populations within systems. Finally we compared the proportions of carbon sources using a Wilcoxon test between parapatric systems. Overall our carbon data does not allow inferences of diet specialization because $\delta^{13}C$ signatures may also be reflective of populations feeding in different habitats. However, it does allow to estimate the respective parapatric habitat contrasts.

Results

Genetic differentiation

Of the 18 microsatellite loci genotyped, one (*Stn209*) was monomorphic in all samples and we discarded it from further analyses. For the remaining loci, the number of alleles per locus varied from 3 to 17. Heterozygosity within population samples, averaged across all loci, varied from 0.470 to 0.625 (mean 0.550, ±0.061 SD; Table 1). Global F_{ST}s, calculated separately for each marker, did not statistically differ between putatively QTL linked and unlinked markers (W = 22, P = 0.301). Comparing the expected heterozygosities of Swiss invasive populations to those from 58 native freshwater populations from across Europe revealed a slight but significant reduction in heterozygosity among the invasive populations in Switzerland (Fig. S1; mean H_e Swiss populations = 0.542, mean H_e European populations = 0.598, $t_{1,58}$ = 2.2, P = 0.035), suggesting that the recent invasion was associated with a slight loss of genetic variation.

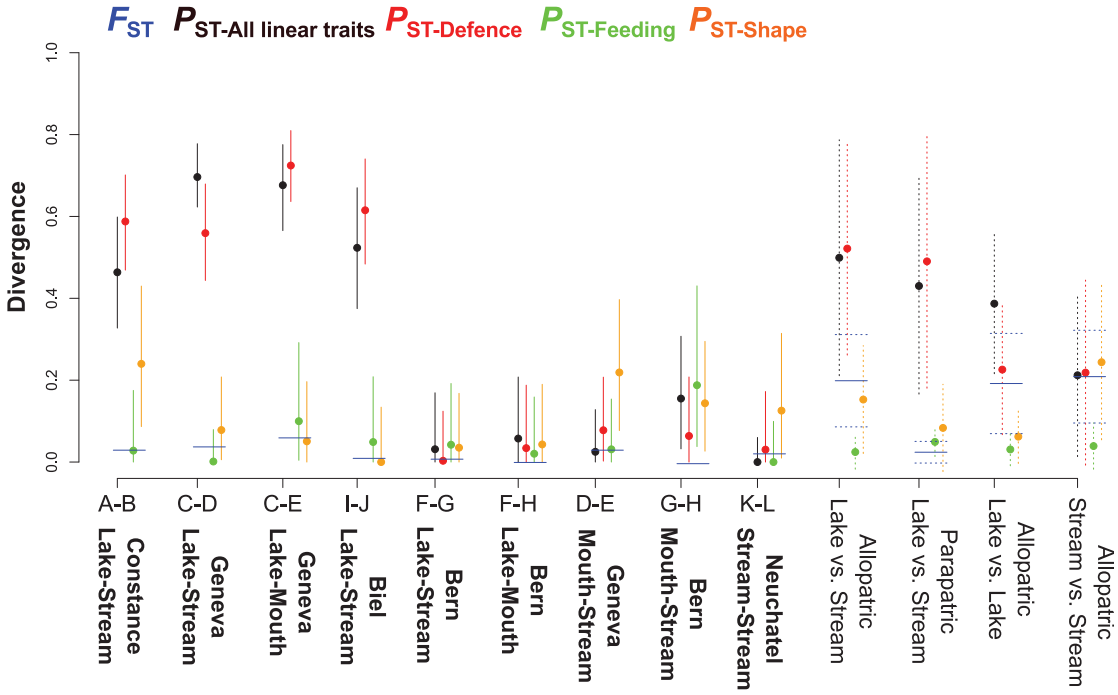

Figure 3: *Degree of parapatric genetic (F_{ST}) and phenotypic (P_{ST}) divergence as well as average degree of divergence among parapatric and allopatric lake-stream as well as allopatric lake-lake and stream-stream comparisons. P_{ST} was based on the individual scores of the first PC axis for either all linear traits combined or separately for defense and feeding traits (see Fig. 2) as well as the first PC for morphometric shape. F_{ST} is given as solid blue horizontal lines, whereas solid vertical bars represent the 95% confidence interval based on a resampling procedure with 1000 replicates (see main text for details). Dashed lines represent the standard deviation of allopatric comparison for both genetic an phenotypic data.*

The degree of parapatric divergence did not statistically differ between unlinked and putatively QTL linked markers (paired *t*-tests: *Stn26*: $t_{1,8}$ = 0.5, P = 0.631; *Stn96*: $t_{1,8}$ = 0.8, P = 0.463; *Stn130*: $t_{1,8}$ = 1.5, P = 0.165; *Stn131*: $t_{1,8}$ = 1.2, P = 0.275; *Stn152*: $t_{1,8}$ = 0.1, P = 0.902; all QTL linked markers combined: $t_{1,8}$ = 0.4, P = 0.669) except for *Stn178* ($t_{1,8}$ = 3.1, P = 0.016). However, in the latter case F_{ST} values were significantly higher for unlinked markers (average F_{ST} = 0.020) than for *Stn178* (F_{ST} = 0.001), which may imply stabilizing selection on this marker. Certainly did this marker not drive parapatric genetic divergence. We consequently pooled all markers for all subsequent analyses. We found that the mean genetic differentiation was significantly lower among populations within a lake system (mean F_{ST} = 0.038 ± 0.051) than among populations from different lake systems (mean F_{ST} = 0.207 ± 0.109; $F_{1,63}$ = 23.16, P < 0.001; Fig. 3). The AMOVA revealed that a much larger proportion of total genetic variance resided among lake systems (19.97%, df = 4, P < 0.001) relative to between habitats within the lake systems (3.04%, df = 7, P < 0.001). This provides a strong basis for our classification of lake-stream habitat pairs sampled within the same lake system as replicates of parapatric population divergence and populations from different lake systems as allopatric (Table S1). We consequently further refer to them as lake and stream populations. The neighbor-joining population tree further supports the classification into parapatric lake-stream populations pairs, showing that, with the exception of the geographically close Biel and Bern systems, samples from contrasting habitats in the same lake system are more closely related to one another than those from similar habitats in different lake systems (Fig. 4a). Populations from the Biel and Bern systems are all closely related such that sister pair relationships within these systems could not be resolved with confidence with our data. However, our data are still most consistent with parallel origins of lake and stream populations even between these geographically adjacent lake systems (Fig. 4a). The population tree shows two main clusters: one containing the two Constance populations, the other containing the three Geneva and the two Neuchatel populations (with 100% bootstrap support in each case). The populations from the Bern and Biel systems fall between these two main clusters, and the Neuchatel populations are intermediate too but closer to the populations from the Lake Geneva system, which reflects the different admixture proportions among three invasive lineages found in these systems (see Lucek *et al.*, 2010). STRUCTURE resolved parapatric populations from different habitats as distinct genetic clusters in both the Lake Constance and the Lake Geneva systems whereas a single genetic cluster was observed in the Biel and Bern systems (Fig. 4b).

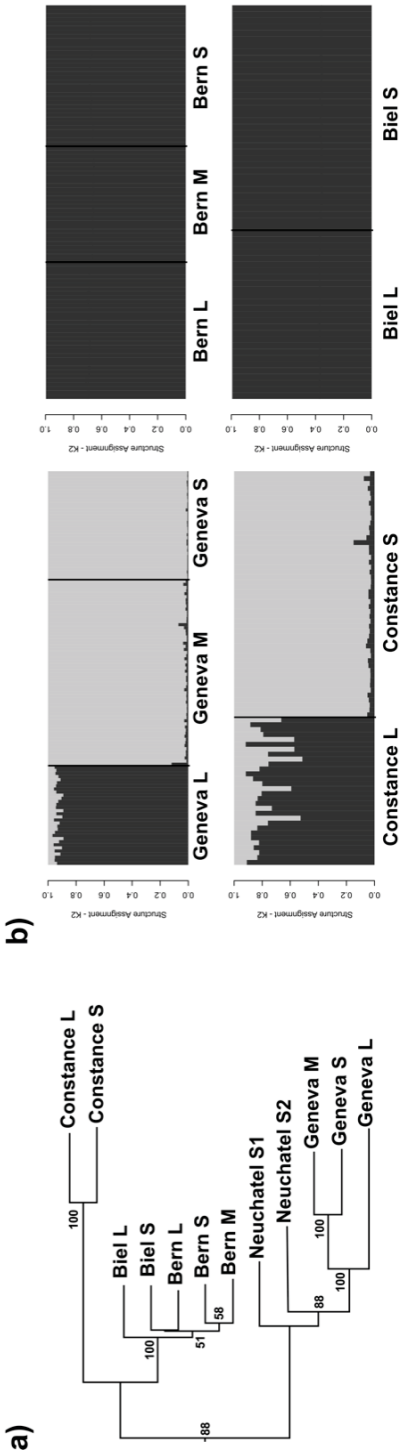

Figure 4: *Genetic differentiation among populations: a) Neighbour-joining tree (midpoint rooted) based on Nei's (1978) unbiased genetic distances amongst populations included in this study, calculated from allele frequencies at 17 microsatellite loci. Numbers beside nodes indicate percent bootstrap support based on 1000 resampling replicates. Bootstrap values below 50% are not shown. b) Genetic clustering inferred using STRUCTURE for each parapatric lake-stream system (Geneva, Constance, Biel, Bern) assuming two genetic clusters using sampling population as a prior. Because the best number of inferred clusters equaled one in Biel and Constance, they are represented as monomorphic clusters.*

Parallelism and nonparallelism of phenotypic differentiation

Parapatric phenotypic differentiation (P_{ST}) differed among systems and traits (Fig. 5). Lake and stream populations differed the most in the Biel and Geneva system, where in each case P_{ST} of nine linear traits exceeded the level of genetic differentiation (F_{ST}), followed by Constance with seven such strongly divergent traits. In the Bern system, P_{ST} of the lake population exceeded F_{ST} only for one trait (UJL), whereas for the comparisons that involved the population

from the stream mouth P_{ST} exceeded F_{ST} more often (three comparisons against the lake population and seven comparisons against the stream population). Similarly the stream mouth population in the Geneva system differed from both the stream and the lake population, but was much more similar to the stream population than to the lake population (mouth-lake: ten comparisons; mouth-stream: three comparisons). Among the two stream populations that we compared in the Neuchatel system, only P_{ST} based on the number of gill rakers exceeded F_{ST}. Overall, anti-predator related defense traits differed most commonly between parapatric habitat contrasts, where lake fish showed elongated spines in comparison to stream and stream mouth populations. Divergence in feeding related traits occurred frequently too, but only gill raker length showed parallel divergence in three lake-stream systems, where lake fish had longer gill rakers than stream and stream mouth fish (Fig. 5) Divergence occurred also in other feeding-related traits but divergence was not repeated among systems. Finally, body depth showed parallel divergence in most lake-stream comparisons with stream fish being deeper-bodied than lake fish, which was equally true for the stream-mouth comparison in the Geneva system.

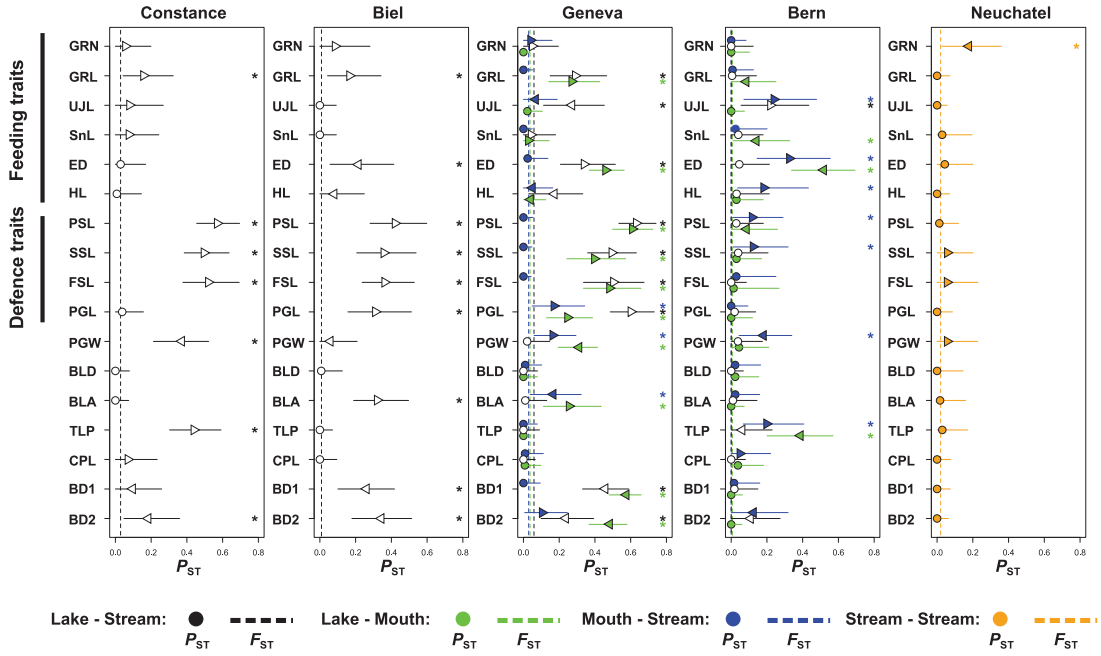

Figure 5: *Parapatric divergence (P_{ST} ±95% CI) for each linear trait (see Fig. 2). Circles depict cases where pairwise comparisons were not statistically significant ($p > 0.05$) based on a t-test, whereas triangles indicate significant pairwise comparisons ($p < 0.05$). The directionality of the triangle further indicates if the first mentioned habitat is larger (pointing right) or smaller (pointing left) than second mentioned habitat for each contrast. Parapatric F_{ST} for each comparison is plotted as dashed vertical line. Cases where $P_{ST} > F_{ST}$ are indicated with an asterisk.*

The first PC axis based either on all linear traits combined, on only defense traits or only feeding related traits, explained 34.6%, 67.9% and 84.2% of the total variation, respectively. The first PC axis for morphometric shape accounted for 31.9% of the total shape variation. None of the PC axes for shape were associated with standard length (all $P > 0.99$). Parapatric P_{ST} based on PC scores using all traits or defense traits only exceeded F_{ST} to a similar degree in

three lake-stream comparisons (Constance, Geneva and Biel) and the lake-mouth comparison in the Geneva system (Fig. 3). Parapatric P_{ST} of the stream-mouth comparison in the Bern system also exceeded F_{ST}, but to a lesser extent. Parapatric P_{ST} using only feeding related traits exceeded F_{ST} only in the stream-mouth comparison within the Bern system. Differentiation in morphometric shape exceeded F_{ST} in the Constance lake-stream comparison and in both stream-mouth comparisons within the Bern and Geneva systems.

The magnitude of phenotypic differentiation between lake populations and between stream populations from different systems, i.e. P_{ST} between allopatric ecotypes, was similarly high as that observed among parapatric ecotypes (PC1 all traits: $W = 33$, $P = 0.615$; PC1 defense traits: $W = 35$, $P = 0.727$; PC1 feeding traits: $W = 63$, $P = 0.071$; PC1 morphometric shape: $W = 27.5$, $P = 0.352$; Fig. 3). Although P_{ST}s derived from the PCAs combining either all traits, defense traits or feeding traits between allopatric populations from the same habitat were on average lower than for parapatric habitat contrasts (Fig. 3), they did not statistically differ between allopatric and parapatric comparisons (lake-lake vs. lake-stream: PC1 all traits: $W = 15$, $P = 0.610$; PC1 defense traits: $W = 18$, $P = 0.257$; PC1 feeding traits: $W = 17$, $P = 0.331$; PC1 morphometric shape: $W = 12$, $P = 0.999$; stream-stream vs. lake-stream: PC1 all traits: $W = 42$, $P = 0.152$; PC1 defense traits: $W = 44$, $P = 0.100$; PC1 feeding traits: $W = 38$, $P = 0.312$).

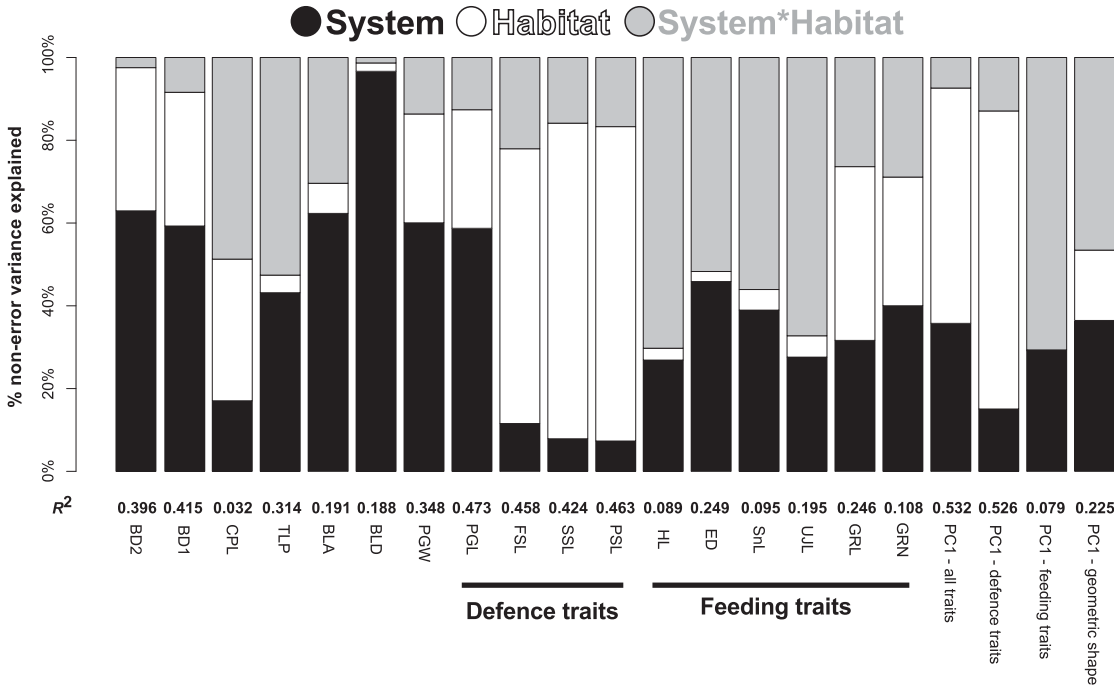

Figure 6: *Percentage of non-error variation explained for the difference among parapatric lake-stream systems (Constance, Geneva, Bern, Biel), the difference between habitats (lake or stream) as well as their interaction for each linear morphometric trait. The R^2 values below each bar further indicate the overall amount of variation explained by each model.*

The trait based ANOVA models all explained a significant amount of variation (average R^2 = 0.288 ± 0.160 SD, Fig. 6; all $P < 0.001$, results not shown), where the lake system explained a significant ($P < 0.05$) amount of variation in all traits except HL and CPL (results not shown; average explained variation by *system*: 38.8% ± 22.3% SD). Differentiation between systems was highest for traits related to body shape or swimming behavior, which was especially true for BLD (Fig. 6). *Habitat*, which is related to parallelism in parapatric lake-stream differentiation, explained on average a similar amount of the phenotypic variation (29.7% ± 26.5% SD; paired *t*-test for the percentage of variance explained by *system* and *habitat*: $t_{1,20}$ = 20.0, P = 0.344). The habitat related component was particularly large in spine lengths, gill raker length and gill raker number as well as body depth. Similarly, the scores of the leading axis of PCA based on either all linear traits or only defense traits, showed a relatively high proportion of habitat dependent variation. Finally, the *system* x *habitat* interaction explained on average 31.6% (± 23.2% SD) of the phenotypic variation, suggesting some system specific component to parapatric lake-stream divergence especially for feeding related traits and to a lesser extent for body shape.

Comparative analysis of lake-stream differentiation

The obtained values for P_{ST} from the Canadian parapatric lake-stream ecotypes differed from the values reported earlier of the same data set (Kaeuffer *et al.*, 2012; reanalyzed in Ravinet *et al.*, 2013) for size corrected shape and gill raker length but not for the number of gill rakers (Fig. S2). This may reflect differences due to the different size correction methods applied in each publication. The values reported here based on size corrected data were closer to the ones reported by Kaeuffer *et al*. (2012) (shape: R^2 = 0.415; gill raker length: R^2 = 0.431) than Ravinet *et al*. (2013) (shape: R^2 = 0.089; gill raker length: R^2 = 0.219; Fig. S2) but that does not change any of the general patterns reported in these studies. Treating all data the same way, we can now compare the extent of parallel and non-parallel divergence among the different systems and studies. The comparative P_{ST} and F_{ST} values showed that whereas the largest differentiation for F_{ST} was observed in Canadian lake-stream systems (Canada vs. Europe: $t_{1,21}$ = 3.1, P = 0.015), the degree of phenotypic differentiation can be as high in Europe as in Canada or higher (Fig. 7, Table 2). The differentiation of parapatric ecotypes in body shape was significantly higher in the Canadian systems ($t_{1,21}$ = 5.1, P = 0.001). Similarly gill raker number ($t_{1,21}$ = 2.2, P = 0.049) showed an increased differentiation in the Canadian systems and also in two out of nine comparisons from Lough Neagh (Ireland), compared to the Swiss and other Irish comparisons. However, with a single exception from Switzerland, the direction of divergence was consistent across all divergent ecotype pairs with lake populations having more gill rakers. Gill raker length was also very consistently divergent between lake and stream ecotypes with lake fish having significantly longer gill rakers in almost all cases. Interestingly, the magnitude of divergence in this trait was not different between Canadian and European systems ($t_{1,21}$ = 0.2, P = 0.811). Finally, Swiss ecotypes exhibited the largest extent of phenotypic divergence in spine lengths. In all European systems with

ecotypic differentiation, lake fish have longer spines than stream fish, albeit the difference is smaller in Ireland. The same divergence is not consistently observed in Canadian lake-stream comparisons, where lake fish can have either longer or shorter spines.

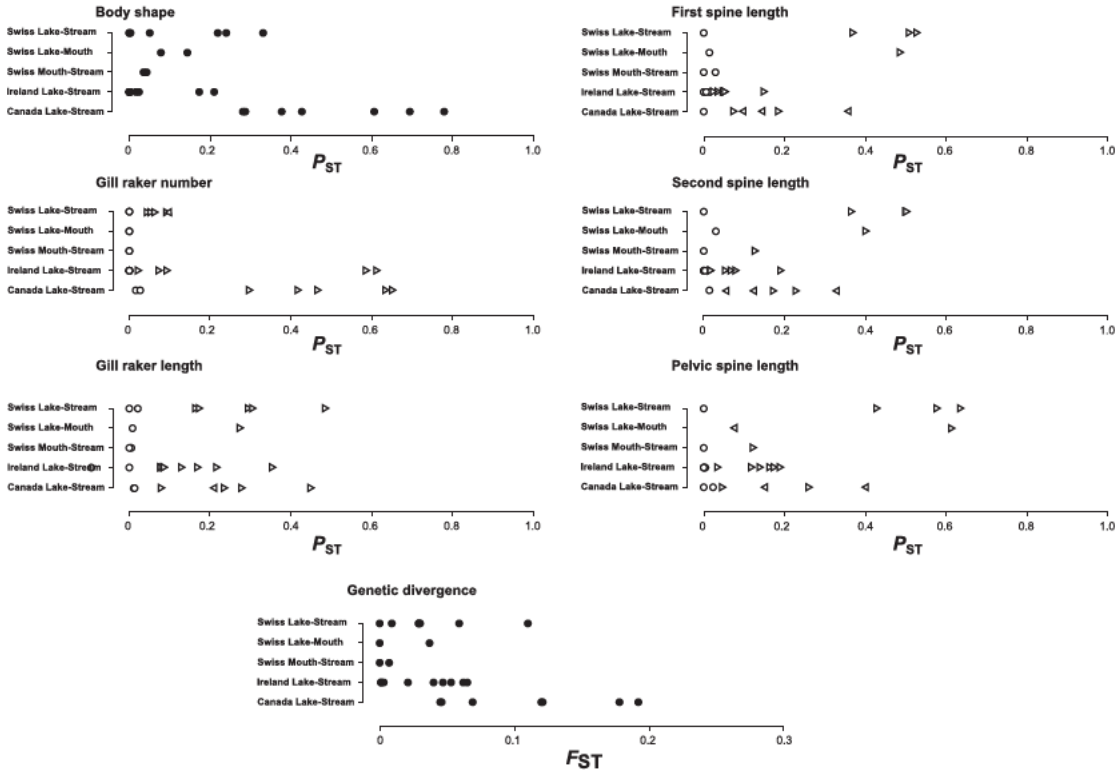

Figure 7: *Comparison of P_{ST} and F_{ST} values between parapatric stickleback ecotype pairs from Canada, Ireland and Switzerland. The directionality of differentiation for linear phenotypic measurements and for the number of gill rakers was statistically inferred using a t-test, where triangles indicate significant ($p < 0.05$) and open circles non-significant ($p > 0.05$) pairwise comparisons. For significant comparisons, the directionality of the triangle indicates if the first mentioned habitat is larger (pointing right) or smaller (pointing left) than the second mentioned habitat for each contrast. Filled circles depict the pairwise P_{ST} for geometric morphometric body shape and the pairwise genetic divergence based on F_{ST}.*

Table 2: *Comparison of pairwise phenotypic (P_{ST}) and genetic (F_{ST}) differentiation between lake-stream ecotypes of stickleback from Switzerland, Ireland and Canada. P_{ST}s are based on the PC1 scores for geometric morphometric body shape, the number of gill rakers or size corrected lengths of gill rakers, first (FSL), second (SSL) or pelvic spine (PSL). See main text for details.*

Region	System	Habitat contrast	Reference	Body shape	# Gill raker	Gill raker length	FSL	SSL	PSL	F_{ST}
Switzerland	Constance	Lake-Stream	this study	0.240	0.064	0.165	0.528	0.503	0.578	0.029
	Geneva	Lake-Stream	this study	0.051	0.054	0.295	0.509	0.500	0.636	0.059
		Lake-Mouth	this study	0.078	0.000	0.275	0.487	0.402	0.614	0.037
		Stream-Mouth	this study	0.219	0.045	0.000	0.000	0.000	0.000	0.029
	Bern	Lake-Stream	this study	0.043	0.000	0.005	0.000	0.000	0.000	0.000
		Lake-Mouth	this study	0.035	0.000	0.000	0.014	0.030	0.075	0.007
		Stream-Mouth	this study	0.144	0.000	0.008	0.029	0.128	0.123	0.000
	Biel	Lake-Stream	this study	0.000	0.093	0.174	0.370	0.366	0.429	0.009
	Neuchatel	Stream-Stream	this study	0.126	0.167	0.000	0.061	0.063	0.013	0.020
	Constance South	Lake-Stream	Berner et al. 2010	0.331	0.097	0.486	-	-	-	0.110
	Constance West	Lake-Stream	Berner et al. 2010	0.003	0.000	0.306	-	-	-	0.030
	Geneva	Lake-Stream	Berner et al. 2010	0.000	0.000	0.021	-	-	-	0.000
Ireland (Lough Neagh)	Ballinderry	Lake-Stream	Ravinet et al. 2013	0.000	0.094	0.078	0.053	0.080	0.175	0.040
	Blackwater	Lake-Stream	Ravinet et al. 2013	0.024	0.023	0.085	0.001	0.000	0.004	0.003
	Crumlin	Lake-Stream	Ravinet et al. 2013	0.000	0.000	0.000	0.000	0.000	0.000	0.021
	Glenavy	Lake-Stream	Ravinet et al. 2013	0.000	0.613	0.355	0.036	0.054	0.190	0.062
	Lower Bann	Lake-Stream	Ravinet et al. 2013	0.016	0.074	0.087	0.025	0.018	0.035	0.001
	Maine	Lake-Stream	Ravinet et al. 2013	0.173	0.587	0.170	0.014	0.017	0.140	0.053
	Moyola	Lake-Stream	Ravinet et al. 2013	0.210	0.000	0.217	0.150	0.191	0.164	0.065
	Sixmilewater	Lake-Stream	Ravinet et al. 2013	0.000	0.000	0.130	0.049	0.068	0.119	0.047

	Upper Bann	Lake-Stream	Ravinet et al. 2013	0.002	0.000	0.079	0.006	0.003	0.000	0.001
Canada, British Columbia	Beaver	Lake-Stream	Kaeuffer et al. 2012	0.427	0.016	0.209	0.097	0.055	0.151	0.192
	Boot	Lake-Stream	Kaeuffer et al. 2012	0.606	0.635	0.236	0.186	0.229	0.261	0.178
	Joe's	Lake-Stream	Berner et al. 2010	0.281	0.418	0.450	-	-	-	0.120
	Misty	Lake-Stream	Kaeuffer et al. 2012	0.779	0.298	0.011	0.075	0.173	0.047	0.121
	Pye	Lake-Stream	Kaeuffer et al. 2012	0.694	0.652	0.279	0.357	0.328	0.400	0.069
	Robert's	Lake-Stream	Kaeuffer et al. 2012	0.377	0.027	0.013	0.144	0.123	0.023	0.045
	Village Bay	Lake-Stream	Kaeuffer et al. 2012	0.287	0.467	0.080	0.000	0.014	0.000	0.046

Consistent differentiation in trophic ecology

Although parapatric ecotypes from Switzerland showed differentiation in their trophic position in all instances, the mean differences were all significantly smaller than 1 ($P < 0.001$; Fig. 8a). This indicates that stickleback populations in all systems share a similar mean trophic position. The direction of divergence in trophic position between lake and stream stickleback varies among systems. The proportion of carbon obtained from a pelagic born source was also highly variable within systems (Fig. 8b). A consistent parallel pattern seen in all sampled lake-stream contrasts suggests that lake populations incorporate a significantly higher proportion of pelagic carbon in their diets than do the stream and stream mouth populations (Constance lake vs. stream: $W = 96$, $P < 0.001$; Geneva lake vs. mouth: $W = 100$, $P < 0.001$; Geneva lake vs. stream: $W = 100$, $P < 0.001$; Geneva mouth vs. stream: $W = 100$, $P < 0.001$; Bern lake vs. mouth: $W = 84$, $P = 0.009$; Bern lake vs. stream: $W = 100$, $P < 0.001$; Bern mouth vs. stream: $W = 86$, $P = 0.005$; Biel lake vs. stream: $W = 100$, $P < 0.001$). The stream mouth population from the Geneva system was more similar to the stream population from higher upstream, whereas the stream mouth population from the Bern system was on average intermediate to the lake and stream populations and showed a high variation among individuals.

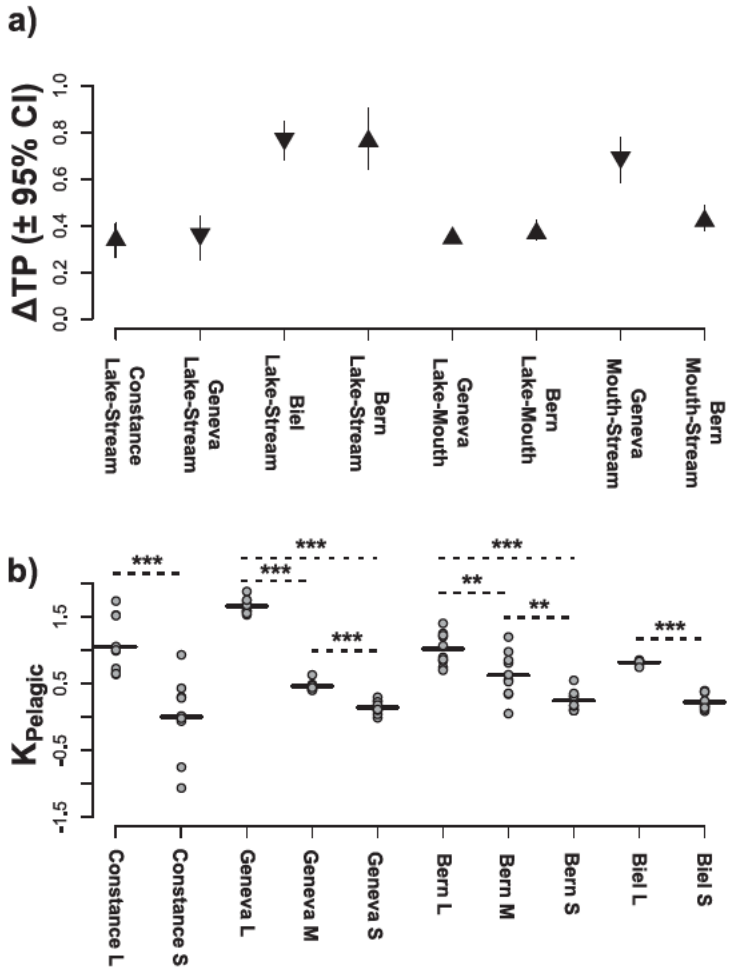

Figure 8: *a) Mean trophic level differences among parapatric stickleback ecotypes in Switzerland. 95% confidence intervals calculated from 10 000 Monte Carlo randomisations. The directionality of the triangle further indicates if the first mentioned habitat is larger (pointing up) or smaller (pointing down) than second mentioned habitat for each contrast. All differences were found to be significantly smaller than 1 showing that ecotypes share a single trophic level (P<0.05). b) Proportion of individual carbon (collection mean indicated by black line) originating from pelagic sources determined using a simple 2-source mixing model. The statistical significance of pairwise differences between parapatric ecotypes are indicated based on Wilcoxon tests (** p < 0.01, *** p < 0.001). See main text for details.*

Evidence for isolation by adaptation

F_{ST} was not significantly predicted by the waterway distance ($F_{1,7}$ = 3.9, P = 0.090) nor by the differences in altitude ($F_{1,7}$ = 0.1, P = 0.740). On the contrary, linear models showed that P_{ST} in BD1 ($F_{1,7}$ = 5.8, P = 0.047), FSL ($F_{1,7}$ = 6.0, P = 0.044), PGL ($F_{1,7}$ = 9.2, P = 0.019), and GRL ($F_{1,7}$ = 7.8, P = 0.030) significantly predict F_{ST} (see Table S2 for details). Although waterway distance and the difference in altitude between our lake, stream and stream mouth populations were significantly correlated ($F_{1,7}$ = 9.3, P = 0.019) none of the models using the altitudinal differences were significant (all P > 0.100, results not shown). Therefore we report only results based on waterway distances. We found support for isolation by adaptation in the form of a significant effect of P_{ST} on F_{ST} when isolation by distance was controlled for in four traits (BD1: $F_{2,6}$ = 8.3, P = 0.018, FSL: $F_{2,6}$ = 6.8, P = 0.029, PSL: $F_{2,6}$ = 6.3, P = 0.034, GRL: $F_{2,6}$ = 8.8, P = 0.016) and in PC1 using all traits ($F_{2,6}$ = 5.6, P = 0.043). For these models adding P_{ST} to waterway distance for predicting F_{ST} led to a substantial increase in R^2 values (Table S2). Two of these results may have been affected by pseudo-replication, where the observed R^2 values were larger than the R^2 values from the resampled models (see Methods): BD1 ($t_{1,8}$ = 2.70, P = 0.027) and GRL ($t_{1,8}$ = 2.45, P = 0.040). Taken together, these results are consistent with predictions of isolation by adaptation, and hence suggest the initiation of the process of ecological speciation (Nosil *et al.*, 2009; Nosil, 2012; Shafer & Wolf, 2013).

Discussion

Many unanswered questions remain regarding the relative importance of genetic, ecological and geographical constraints to adaptive evolutionary diversification of lineages, and often times empirical tests lag behind theory (Gavrilets & Losos, 2009; Nosil, 2012; Abbott *et al.*, 2013). Unresolved issues are related to the balance between adaptive divergence and gene flow and to the general relationship between these forces. Gene flow may often constrain adaptive divergence such that populations would be more divergent if gene flow was absent (Garant *et al.* 2007, Räsänen & Hendry, 2008). Gene flow, however, can also promote adaptive divergence (Garant *et al.*, 2007; Räsänen & Hendry, 2008; Edelaar & Bolnick, 2012; Abbott *et al.*, 2013). Invasive species are useful

models to address questions about the onset of adaptive diversification (Prentis *et al.*, 2008; Westley, 2011). Using the recent invasion of Swiss waterways by stickleback, where populations occupy a wide range of habitats and harbor much increased trait variation relative to individual source populations in their native range (Lucek *et al.*, 2010)**,** we addressed some of these questions regarding the onset of diversification. We asked if the wide habitat occupation and increased trait variation were associated with ecotypic differentiation between major habitats. We assessed whether or not the direction of differentiation was repeatable, whether it was predictable by the habitat contrast and if it was constrained by gene flow. Hence we tested for ecology-driven evolutionary differentiation within the invasive range, which may be considered the first phase in adaptive radiation (Schluter, 2000). Finally, we evaluated whether phenotypic divergence predicted genetic differentiation at neutral marker loci, which would indicate the initiation of the process of ecological speciation (Schluter, 2000; Nosil, 2012).

Replicated parapatric ecotypic differentiation among Swiss lake-stream systems

In stickleback, habitat dependent phenotypic divergence between lake and stream populations has been shown to occur through adaptive phenotypic plasticity (e.g. Wund *et al.*, 2008; Leaver & Reimchen, 2012) as well as through selection on standing genetic variation (Deagle *et al.*, 2012). The relative importance of each may depend on the investigated trait and population. Especially anti-predator related traits often diverge between contrasting habitats as a consequence of divergent predation regimes (Reimchen, 1980; 1994; Marchinko, 2009). Similarly, adaptation to different feeding strategies is also thought to drive ecological divergence between benthic feeding stream populations and often more limnetic feeding lake populations (Berner *et al.*, 2008; Kaeuffer *et al.*, 2012). Body shape and especially body depth may diverge due to habitat related differences in flow regimes and requirements for swimming behavior (Bergstrom, 2002; Wark & Peichel, 2010; Hendry *et al.*, 2011). Many of the underlying traits that experience divergent selection have been shown to be heritable, including gill raker numbers (Hagen, 1973; Hermida *et al.*, 2002), spine length (Dingemanse *et al.*, 2009) and body depth for populations from Canada (Berner *et al.*, 2011). Feeding related head shape on the other hand seems rather plastic in those populations (Wund *et al.*, 2008; Berner *et al.*, 2011). In Swiss stickleback, experimental work, focusing on feeding related divergence, suggests a combination of both heritable and plastic components. In particular, feeding related head shape is rather genetically determined and body depth is rather plastic (Lucek et al. *submitted*).

Here we investigated parapatric populations within five lake systems in Switzerland that differ from most of the studied lake-stream ecotype pairs from elsewhere in three key features: First, the time available for ecotypic divergence, with our lake-stream pairs ranging in age between <90 and 140 years, whereas most other studies investigated much older lake-stream pairs (e.g. Berner *et al.*, 2009). Ecotype formation within freshwaters on a similarly recent contemporary time scale has only been investigated in two other cases: Two other Swiss

population contrasts in the Constance and Geneva system (Berner *et al.*, 2010) and in California (Hendry *et al.*, 2013). Second, the evolutionary history of Swiss populations, which derive most likely only from divergent freshwater lineages that independently colonized different European river systems post-glacially (Mäkinen & Merilä, 2008; Lucek *et al*., 2010). This contrasts with the lake-stream systems that have been studied in Canada that evolved directly from marine ancestors, possibly through double invasion processes (Taylor & McPhail, 2000; Schluter & Conte, 2009; Jones *et al*., 2012b). Hence, the observed divergence among Swiss systems evolved via selection on standing genetic variation from freshwater populations rather than from an ancestral marine population. Finally, the magnitude of the habitat contrasts, where most of our studied lakes are much larger and deeper, and in that sense more marine-like, than formerly studied lakes (Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b).

Despite being relatively young, we observe significant genetic differentiation between parapatric lake, stream and stream mouth populations in the Constance, Geneva and the Biel system (Table S1) but not in the Bern system. In addition the two stream populations from different tributaries of Lake Neuchatel are genetically differentiated too (Table S1). Parapatric ecotypes are genetically most closely related to each other within lake systems except perhaps among the closely related Bern and Biel systems (Fig. 4a). This suggests that adaptation to the distinct habitat contrasts studied here occurred in parallel in at least three instances, i.e. the Constance, Geneva and Bern/Biel systems. We find that overall morphological divergence exceeds the expectations from neutral genetic differentiation in most parapatric contrasts between different habitats. This is also true specifically for anti-predator related morphology, gill raker lengths and body depth (Fig. 5). All of these traits are known to experience habitat dependent divergent selection in Canada (Reimchen, 1994; Robinson, 2000; Wark & Peichel, 2010). This suggests that divergent selection between habitats has driven phenotypic divergence since the colonization of Swiss waterways. In contrast to the linear trait measurements, significant divergence in overall body shape occurs only in some comparisons (Fig. 3). Together, these results imply that – independent of the lake system – the two habitat types induce analogous divergent selection pressure, related to predation and feeding ecology, leading to similar and consistent ecotypic divergence among stickleback populations. This is especially remarkable given that some of our studied ecotype pairs (Constance versus Geneva) represent the descendants of distantly related and phenotypically very different European lineages (Lucek *et al.*, 2010). Hence the parallelism that we observed between these systems trumped historical contingency, making our results a clear example of independent parallel evolution.

In contrast to the observed habitat dependent phenotypic divergence, the relative trophic position of parapatric ecotypes in the food web based on nitrogen isotopic ratios was similar, independent of habitat (i.e., $<\pm 1$ trophic units (Post, 2002). This suggests conservatism of stickleback trophic position between different habitats (Fig. 8). However, the proportion of carbon emanating from a pelagic source may suggest a trophic differentiation within this trophic position in each lake-stream system. In all parapatric contrasts, lake populations showed a significantly higher mean proportion of carbon derived

from pelagic sources than their associated stream or stream mouth populations. Such differences are consistent with individuals from the lake feeding more pelagically on zooplankton and using fewer littoral-benthic born dietary sources relative to their stream and stream-mouth counter-parts. Our findings are in line with studies on diversification within lakes along the benthic-limnetic axis (Snowberg & Bolnick, 2008; Matthews *et al.*, 2010). Yet they differ from Kaeuffer *et al.* (2012), who report the opposite pattern for diversification along the lake-stream axis potentially as a result of different flow regimes among their studied streams. Stomach content data further support dietary differentiation between lake and stream stickleback in Switzerland (Gross & Anderson, 1984; Moser *et al.*, 2012; Lucek *et al.*, 2012a).

Overall, we observed the largest phenotypic contrasts in the three systems where we sampled populations from very different habitats, namely little streams versus the shores of the very large and deep lakes Constance, Geneva and Biel. Much smaller differences were observed between the smaller and shallow man-made Lake Wohlen and associated streams and between two streams in the Neuchatel system. The strongest genetic structure is also seen in two of the systems with the largest habitat contrasts, Constance and Geneva (Fig. 4b).

Parallelism and nonparallelism of parapatric divergence

Because the occurrence and extent of parapatric population divergence depends on the underlying environmental and selective gradients (Endler, 1977; Doebeli & Dieckmann, 2003), parallel evolutionary divergence is only expected when the selective regimes are very similar among systems (Kaeuffer *et al.*, 2012). Cases of parapatric lake-stream stickleback systems provide both evidence for parallelism and nonparallelism in the realized trait-specific divergence that occur both on smaller geographical scales as well as between continents (Hendry & Taylor, 2004; Berner, 2009; Berner *et al.*, 2010; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b). Cases of nonparallelism may arise through different selective regimes in similar habitats (Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b), genetic constraints (Berner *et al.*, 2010) or the evolutionary time for divergence (Berner *et al.*, 2010; Hendry *et al.*, 2013).

Overall, our results suggest strong parallelism among Swiss ecotype pairs in habitat dependent differentiation for spine lengths and the PC axis combining anti-predator related traits (Fig. 6). This is remarkable as studies of similar ecotypes from elsewhere in the world did not find strong parallelisms for defence related traits (Deagle *et al.*, 2012; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b). This could imply that selective regimes among different Swiss waterways are more similar than those among waterways elsewhere. Similar selective regimes are furthermore suggested also by the parallelism in gill raker length and number, as well as body depth, but these are shared also with ecotype pairs from elsewhere (Kaeuffer *et al.*, 2012). On the other hand, especially morphometric shape and linear traits that are linked to body shape and swimming behavior show a higher system specific variation than for example spine lengths, which may point to lineage specific historical contingencies.

Finally the system and habitat interaction that accounts for the combined effect of system related historical contingency and parallel ecotypic divergence is highest for feeding related traits.

Amongst the previous studies on lake-stream divergence in stickleback, the strongest parapatric divergence was observed in British Columbia (Canada) for morphometric shape, gill raker numbers, as well as, for genetic divergence (Berner *et al.*, 2010; Kaeuffer *et al.*, 2012). Less divergence was found in much younger ecotype pairs from Switzerland (Berner *et al.*, 2010). In the latter case, the authors suggested that time for divergence and genomic constraints might be responsible for the relatively minor phenotypic divergence. It is indeed possible that European populations are genomically constrained relative to Canadian populations because some of the genetic variation that is found in the Pacific lineage was lost upon colonization of the Atlantic, and it is this Atlantic marine lineage from which the European populations are derived (Jones *et al.*, 2012a). In accordance with these earlier findings, we find that phenotypic divergence in morphometric shape and gill raker number is significantly lower in European populations than among the Canadian systems (Fig. 7). In contrast with these earlier findings though, we find that parapatric divergence in gill raker lengths is quite similar on both continents, where Swiss systems can be as divergent as Canadian systems. Differences in genetic constraints affecting variation in gill raker length or in the ability to express phenotypic plasticity for this trait between stickleback from the Pacific coast of North America versus the Atlantic derived European populations may account for the observed difference. Alternatively, differences in the selective regimes between lake-stream contrasts in Canada and Europe could explain the observed pattern although this seems unlikely. Most importantly, we find the strongest phenotypic divergence for anti-predator related traits in Swiss systems, much stronger than that reported in either Canadian or Irish systems. Perhaps this is explained by the larger habitat contrasts in the Swiss systems, where our studied lakes Constance, Geneva and Biel are generally larger and deeper than the lakes studied in Canada. The predator-driven selective regimes in these lakes may resemble a marine-like environment, where increased spine lengths are favored (Reimchen, 1994).

Evidence for ecological speciation

The causal relationship between adaptive divergence and limits to gene flow is difficult to establish (Garant *et al.*, 2007; Räsänen & Hendry, 2008; Shafer & Wolf, 2013). Positive correlations can be interpreted either as gene flow constraining adaptive divergence or *vice versa*. One way to test the role of adaptive divergence is to compare multiple pairs of populations that differ in their opportunities for gene flow (Nosil & Sandoval, 2008; Berner *et al.*, 2009; Stelkens & Seehausen, 2009a; Moser *et al.*, 2012), as we have done here. Even though the general relationship between gene flow and adaptive divergence may still be difficult to resolve unambiguously (Räsänen & Hendry, 2008), in the present case, gene flow does not appear to impose much constraint on adaptive divergence for the traits that show strong parallelism in parapatric divergence across lake-stream systems. Conversely differentiation at microsatellite loci is

explained by a combination of both geographic distance and phenotypic divergence (Table S2). The use of neutral genetic markers to infer ecological speciation has some potential caveats. First, neutral markers can be affected by random processes such as drift, leading to the detection of false positive cases for ecological speciation. This applies especially when gene flow is low and divergence among all populations is high in the absence of divergent selection (Thibert-Plante & Hendry, 2010). Secondly, differences at neutral genetic markers may not necessarily reflect gene flow if a system is not at equilibrium. On the one hand, founder effects may cause stronger genetic differentiation than expected at equilibrium. Therefore if two populations originate from two independent colonization events, founder effects or pre-existing genetic differentiation between the source populations could result in an underestimation of gene flow (Labonne & Hendry, 2010). On the other hand, if a large population splits into two in the absence of founder effects, the level of genetic differentiation at neutral genetic markers may be lower than at equilibrium and hence overestimating gene flow (Hendry *et al.*, 2000).

Albeit founder events may account to some degree for the allopatric genetic divergence among our studied lake-stream systems, the observed parapatric genetic divergence within each system should not be affected, as they seem to each originate from a single founder event (Fig. 3). Similarly, initial founder events seem to play only a minor role, as the genetic variation was only slightly reduced in comparison to other European populations (Fig. S1). In addition, testing for isolation by adaptation, only the models for spine and gill raker length as well as body depth were significant, which are traits that are known to experience habitat dependent divergent selection. Thus, it appears that adaptive divergence especially for anti-predator related traits and potentially gill raker length and body depth have lessened the homogenizing effects of gene flow by increasing the reproductive isolation between ecotypes. Restrictions to gene flow through divergent natural selection and phenotypic divergence, over and above the limitations imposed by geographic distance is furthermore indicated because phenotypic divergence among parapatric lake-stream contrasts is no less than among allopatric lake-stream contrasts, despite much smaller F_{ST} (Fig. 3). This is a prediction of the early stages of ecological speciation and, when replicated many times within a lineage, marks the potential onset of adaptive radiation (Schluter, 2000; Nosil *et al.*, 2009; Nosil, 2012; Shafer & Wolf, 2013). Our study therefore adds to the rare – but growing – evidence for the rapid evolution of partial reproductive isolation (e.g. Hendry *et al.*, 2000; Rolshausen *et al.*, 2009, see Nosil, 2012 for a review).

Conclusions

Taken together, we show that the very recent invasion of Switzerland by threespined stickleback is associated with the initiation of eco-morphological differentiation between populations inhabiting different major habitats, large lake and stream, that may potentially lead to ecological speciation and adaptive radiation. We show that the phenotypic axes of divergence are parallel and predictable for some trait categories in replicate lake-stream systems that

evolved independently after colonization by distinctly different lineages. Most notably, we find patterns consistent with the hypothesis that the phenotypic divergence between parapatric ecotypes restricts gene flow, signaling the earliest steps towards adaptive ecological speciation. The general implications of our results are twofold. First, they suggest that parapatric ecotype formation can occur relatively fast and along parallel phenotypic trajectories in independent cases with similar environmental contrasts. Secondly, phenotypic parallelism in habitat dependent divergence is seen despite different evolutionary histories of the different populations, suggesting a strong and consistent habitat dependent selective regime.

Acknowledgements

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Supplement

Table S1: *Table of pairwise F_{ST} among all population pairs. Parapatric comparisons within lake systems are boxed. F_{ST} with $p < 0.05$ are given in bold.*

		Constance		Geneva			Bern			Biel		Neuchatel	
		L	S	L	M	S	L	M	S	L	S	S1	S2
Constance	L	-											
	S	**0.029**	-										
Geneva	L	**0.352**	**0.372**	-									
	M	**0.377**	**0.394**	**0.037**	-								
	S	**0.372**	**0.386**	**0.059**	**0.029**	-							
Bern	L	**0.136**	**0.155**	**0.258**	**0.261**	**0.263**	-						
	M	**0.155**	**0.163**	**0.253**	**0.258**	**0.259**	**0.007**	-					
	S	**0.144**	**0.151**	**0.267**	**0.271**	**0.277**	-0.001	-0.004	-				
Biel	L	**0.144**	**0.152**	**0.257**	**0.260**	**0.264**	0.004	**0.008**	0.006	-			
	S	**0.143**	**0.162**	**0.252**	**0.259**	**0.265**	0.001	0.005	0.000	**0.009**	-		
Neuchatel	S1	**0.338**	**0.356**	**0.050**	**0.067**	**0.066**	**0.234**	**0.233**	**0.243**	**0.231**	**0.231**	-	
	S2	**0.303**	**0.327**	**0.043**	**0.063**	**0.066**	**0.192**	**0.191**	**0.202**	**0.191**	**0.189**	**0.020**	-

Table S2: *Summary table of general linear models describing the amount of variation (R^2) and the respective p value for each model. Statistically significant p values (i.e. $p < 0.05$) are highlighted in bold, cases where $0.05 < p < 0.1$ are given in italics. Models are based on pairwise parapatric P_{ST} values that was calculated separately for each trait or on PC scores, where different sets of traits were combined. Either the parapatric waterway distance or the difference in altitude was used to estimate the geographic distance between sampling sites.*

Model			HL	ED	BD1	BD2	CPL	FSL	SSL	PSL	PGW	PGL	TLP
$F_{ST} \sim$ *waterway*	R^2	0.356											
	p	0.090											
$F_{ST} \sim P_{ST}$	R^2		0.011*	0.011*	**0.453**	0.163	0.036	**0.462**	*0.392*	*0.437*	0.043*	**0.567***	0.068
	p		0.786	0.789	**0.047**	0.281	0.626	**0.044**	*0.071*	*0.052*	0.591	**0.019**	0.499
$F_{ST} \sim P_{ST}$ + waterway	R^2		0.368*	*0.449*	**0.736***	*0.514*	0.369	**0.693**	*0.621*	**0.677**	0.415*	*0.601*	0.369
	p		0.116	*0.096*	**0.029**	*0.081*	0.115	**0.039**	*0.055*	**0.042**	0.105	*0.060*	0.115

Model			BLA	BLD	SnL	UJL	GRL	GRN	PC1 - all traits	PC1- defence traits	PC1 - feeding traits	PC1 – geometric shape
$F_{ST} \sim$ *waterway*	R^2	0.356										
	p	0.090										
$F_{ST} \sim P_{ST}$	R^2		0.003*	0.293	0.001*	0.007*	**0.512**	0.033	*0.406**	*0.438**	0.020*	0.028*
	p		0.890	0.133	0.930	0.831	**0.030**	0.640	*0.065*	*0.052*	0.716	0.666
$F_{ST} \sim P_{ST}$ + waterway	R^2		0.365	*0.545*	0.376	0.386*	**0.746***	0.379	**0.651**	*0.612*	0.428*	0.357*
	p		0.116	*0.073*	0.114	0.111	**0.027**	0.113	**0.048**	*0.057*	0.102	0.118

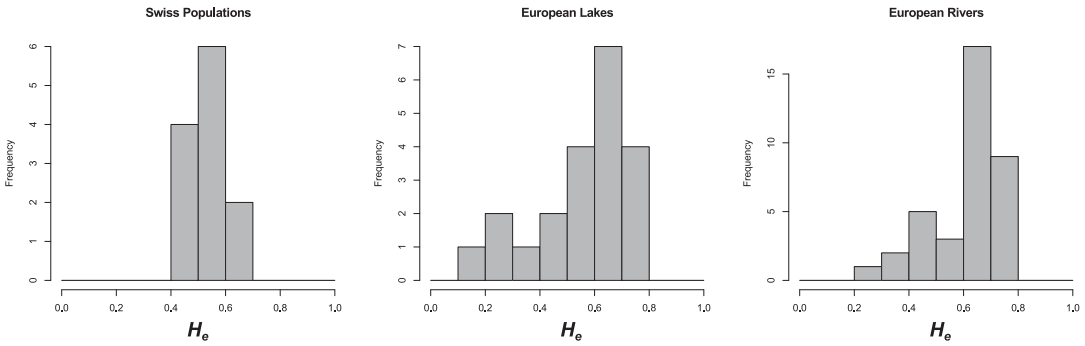

Figure S1: *Expected heterozygosities (H_e) of the European freshwater populations of stickleback studied by Mäkinen et al. 2006 and the Swiss populations in this study.*

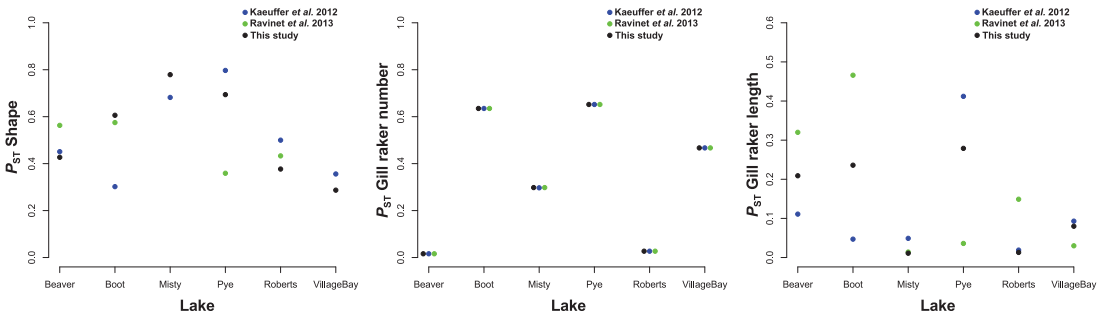

Figure S2: *Pairwise P_{ST} values for either body shape, the number of gill raker or gill raker length between lake and stream habitats for six lakes from British Columbia, Canada. The P_{ST} values were taken from Kaeuffer et al. 2012, Ravinet et al. 2013 and this study and are based on the same data set, which experienced different size correction.*

Supplementary methods:

Genomic DNA was extracted from fin tissue sample, using either a Qiagen BioSprint 96 robot with the Qiagen Blood Extraction kit (Qiagen, Switzerland) or the Promega Wizard DNA extraction kit (Promega, Switzerland). Extracted DNA was diluted to 30 ng per µl. 20 microsatellites, distributed across the genome, were selected from the stickleback linkage map (Peichel et al. 2001) and amplified in five multiplexing sets (Table S1).

The polymerase chain reaction (PCR) reactions consisted of 5 µl Qiagen Multiplexing Solution (Qiagen, Switzerland), 1 µl primer mix (Table S3), 3 µl dH_2O and 1 µl DNA per reaction. The PCR started with 15 min at 95°C followed by 37 cycles with 94°C for 30 seconds, 57°C for 90 seconds and 72°C for 60 seconds with a final elongation at 60°C for 30 minutes. PCR products were 1:10 diluted and visualized on a CEQ 8000 (Beckman Coulter, Switzerland) following the manufacturers instruction. Alleles were scored using the CEQ software and checked for Hardy-Weinberg Equilibrium using Micro-Checker (Oosterhout et al. 2004). Two problematic microsatellites (STN 122 and 199) were then omitted from all further analyses.

Table S3: *Microsatellites used in this study with their respective multiplexing set, the fluorescent and concentration used. Primers and the position in the genome, i.e. linkage group, as well as their putatively linked QTL phenotype were obtained from Peichel et al. 2001.*

Marker	Linkage group	Multiplexing set	QTL	Fluorescent	µl per reaction [10 µM]
STN 10	1	1		Blue	0.1
STN 209	26	1	Lateral plates	Blue	0.1
STN 130	11	1	2nd dorsal spine	Green	0.34
STN 195	20	1		Black	1.5
STN 37	4	2		Blue	0.25
STN 177	16	2		Green	0.68
STN 19	2	2		Black	6
STN 32	3	3		Blue	0.4
STN 152	13	3	Lateral plates	Black	2.25
STN 26	2	4	1st dorsal spine	Green	0.25
STN 132	11	4		Blue	0.25
STN 57	5	4		Black	2.1
STN 110	9	4		Black	1
STN 122	10	4		Green	1.2
STN 82	7	5		Black	0.5
STN 131	9	5	Gill rakers	Black	1
STN 178	16	5	Gill rakers	Green	0.4
STN 96	8	5	2nd dorsal spine	Blue	0.3

Chapter 4

Evidence of adaptive evolutionary divergence during biological invasion

Kay Lucek, Arjun Sivasundar, Ole Seehausen

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Abstract

Rapid phenotypic diversification during biological invasions can either arise by adaptation to alternative environments or by adaptive phenotypic plasticity. Where experimental evidence for adaptive plasticity is common, support for evolutionary diversification is rare. Here, we performed a controlled laboratory experiment using full-sib crosses between ecologically divergent threespine stickleback populations to test for a genetic basis of adaptation. Our populations are from two very different habitats, lake and stream, of a recently invaded range in Switzerland and differ in ecologically relevant morphological traits. We found that in a lake-like food treatment lake fish grow faster than stream fish, resembling the difference among wild type individuals. In contrast, in a stream-like food treatment individuals from both populations grow similarly. Our experimental data suggest that genetically determined diversification has occurred within less than 140 years after the arrival of stickleback in our studied region.

Introduction

Numerous cases of rapid phenotypic diversification during biological invasions are known (Ghalambor *et al.*, 2007; Pfennig *et al.*, 2010). Many are thought to have arisen through adaptive phenotypic plasticity as a consequence of different selection pressures experienced during range expansion. Plasticity provides the possibility for rapid colonisation of new niches by expressing adapted phenotypes readily in different environments (Ghalambor *et al.*, 2007; Pfennig *et al.*, 2010). On the other hand, genetic divergence between populations based on alternative alleles of genes underlying ecologically relevant phenotypes can arise rapidly through natural divergent selection and such divergence can itself be enhanced by plasticity. However, few examples exist for evolutionary or adaptive diversification, defined here as divergence in heritable traits, in such evolutionarily young systems (Vellend *et al.*, 2007). If phenotypic diversification emerges mainly through plasticity, diversification might be impeded between ecologically differentiated phenotypes, because selection can be dampened (Pfennig *et al.*, 2010). In addition, the processes causing diversification during a biological invasion resemble the processes involved in adaptive radiations at an early stage (Yoder *et al.*, 2010). Hence, an identification of one of the abovementioned processes may shed light on the evolutionary pathways leading to apparently adaptive phenotypic diversification. Controlled laboratory experiments in which treatments differ in one or more key factors with all other conditions being the same, provide a powerful method to distinguish between genetically based divergence and plasticity in phenotypically differentiated populations (Kawecki & Ebert, 2004; Sharpe *et al.*, 2008).

A suitable candidate system for studying recent ecological diversification during biological invasion is the threespine stickleback (*Gasterosteus aculeatus*), in Switzerland. In its native range this fish species has repeatedly evolved divergently adapted freshwater ecotype pairs within the last 12,000 years. Many of the observed phenotypic shifts have been attributed to ancestral plasticity in the marine population (Wund *et al.*, 2008). However, in some of these systems, indications for a genetic basis of adaptive diversification have been found (Day *et al.*, 1994; Schluter, 1995). These show fitness trade-offs between the differentiated coexisting sympatric ecotypes (Day *et al.*, 1994; Schluter, 1995) and to a lesser degree in parapatric ecotypes (Hendry *et al.*, 2002). In its invasive range in Lake Constance, Switzerland, ecologically distinct populations occur, living either in the lake or in streams and which differ in their trophic niches (Berner *et al.*, 2010). The stream dwelling populations feed mainly on benthic macroinvertebrates, whereas the lake dwelling population feeds mainly on zooplankton (Figure 1) and has longer gill rakers, suitable to filter small planktonic prey (Berner *et al.*, 2010). Stable isotope data further supports ecological diversification into a mainly zooplankton feeding lake ecotype and a mainly benthos feeding stream ecotype (Lucek *et al.,* 2013). This ecological diversification is striking as stickleback have only been introduced about 140 years ago in the Lake Constance region, deriving from a single East European genetic lineage as inferred from mitochondrial DNA (Lucek *et al.*, 2010). Neutral genetic markers further suggest genetic differentiation between the phenotypically divergent populations in this region (Lucek *et al.*, 2010; Berner *et al.*, 2010).

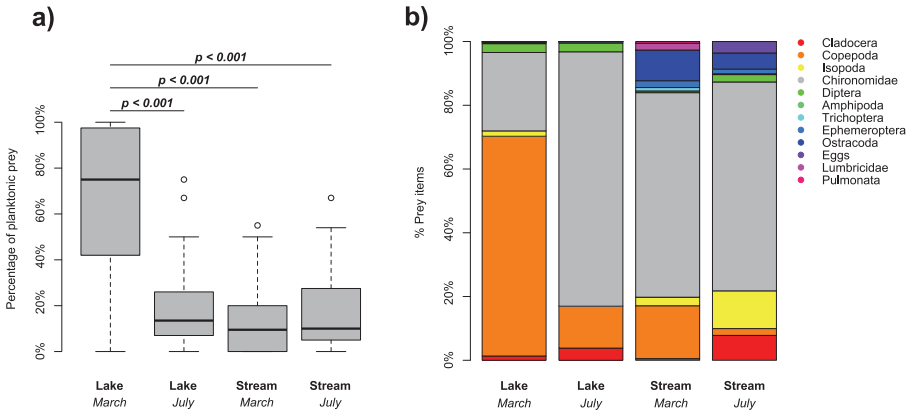

Figure 1: *a) Percentage of planktonic prey in the stomachs of adult stickleback caught either at the lakeshore or stream habitat before the beginning of the breeding season (March 2009) and during the breeding season (July 2007). Indicated significances are based on post hoc t tests for a generalized linear model among sampling events (see text for details). b) Relative abundance of prey items in the stomach of all fish pooled per sampling event.*

Here we test if the phenotypic and ecological differentiation that we observe in the invasive range of sticklebacks in Switzerland can be attributed to evolutionary divergence due to adaptation to different feeding regimes, which represents a major axis of divergence in our study system (Figure 1). Using a controlled laboratory experiment with full-sib F1 families, we test for differences in relative growth rates, measured as the overall difference in body size over time between a lake and a stream population when fed on either a "lake-like" (limnetic) or "stream-like" (benthic) diet. Evolutionary divergence is indicated if trait differences are maintained between the experimental groups. In addition, reduced growth would suggest adaptive differentiation if it is found in at least one population when fed on "foreign" food (Kawecki & Ebert, 2004). Alternatively if phenotypic diversification derives mainly from adaptive plasticity, individuals from both environments raised under identical conditions would express the phenotype that matches the laboratory rearing environment.

Here, we use the increase in body size over time, which is related to the growth rate as a relative measure of fitness, since the wild populations studied here differ in their growth trajectory (Figure 2). This could reflect divergent adaptation due to e.g. different predation (Frommen *et al.*, 2011) or feeding (Schluter, 1995; Bolnick & Lau, 2008) regimes. We furthermore focused on body size as this trait can be easily estimated with little handling effort, which minimizes stress and reduced performance. We focus on the comparison of different ecotypes within an experimental feeding regime rather than comparing the regimes themselves because it provides a direct test for directional selection within each habitat, which may differ between habitats (Kawecki & Ebert, 2004).

Material and Methods

Pre-experimental data collection

In a preliminary study in July 2007 (Lucek *et al.*, 2010), wild adults from Lake Constance, Switzerland (47°29'02''N, 9°33'35''E) and from a stream, about 25 km upstream of the lake (47°19'33''N, 9°34'41''E) were sampled using minnow traps and by hand netting (N_{Lake} = 14, N_{Stream} = 32). Additional samples were obtained for both habitats in March 2009 (N_{Lake} = 25, N_{Stream} = 22). All fish were sacrificed in the field with an overdose of anaesthetic MS-222 and preserved in 95% ethanol for further analysis.

For each individual, stomachs were extracted and all food items were counted using a dissection microscope. Food items were assigned to the following taxonomic classes: *Amphipoda, Chironomidae, Cladocera, Copepoda, Diptera* imagos, *Ephemeropera, Isopoda, Lumbricidae, Ostracoda, Pulmonata, Trichoptera,* and stickleback eggs. The percentage of planktonic prey was then calculated as the fraction of *Cladocera* and *Copepoda* to the total number of all food items present for each individual. Sampling events were statistically compared with a generalized linear model (GLM) assuming a quasibinomial distribution to account for over dispersion of the data. Pairwise significances were established using post hoc *t* tests. Two lake individuals from 2009 with empty stomachs were excluded. After extraction of the stomachs, all individuals were stained with formaldehyde and alizarin red to count their lateral plates for a different study (see Lucek *et al.*, 2010 for details).

Experimental fish collection

Ripe individuals from the same sites as for the preliminary study were sampled in May 2010. Pairs (one male and one female) from the same source population were kept in individual 60 x 30 x 40 cm aquaria containing sand substrate, natural nesting material as well as a filtering and aerating system. After a successful spawning event the parental fish were removed. In addition to the individuals used for the crosses, a random subset of the wild population was preserved (N_{Lake} = 91, N_{Stream} = 49). These individuals were measured for their standard length. In addition, both otoliths, calcium carbonate structures in the inner ear that show seasonal rings, were extracted for each individual. Winter rings were counted at 40x magnification with a microscope to estimate the age of each individual. Age could not be determined for the individuals used in the preliminary study since the staining process dissolves calcium structures. Standard length was compared between habitats and age classes using an ANOVA with a Tukey's HSD post hoc test. Overall differentiation was estimated with an ANOVA with age as a random factor to account for differences among age classes.

Experimental setup and husbandry

Fertilized eggs were kept aerated in each tank. Eggs with fungal infection or dead embryos were removed daily. Two thirds of the water in each tank was

replaced with well water every two days throughout the experiment. All hatched individuals were fed with *Artemia sp.* nauplii for the first five weeks after hatching. Between weeks four and five, small nematodes (*Panagrellus sp.*) were also provided. After this time, six stream families and seven lake families were randomly chosen. Each full-sib family was split into two subsets of 18-20 individuals each, one group being assigned to a "limnetic" type food regime, and the other to a "benthic" type food regime from week six onwards. The provided food items represent the main prey items eaten in the wild, based on the pre-experimental stomach content analyses (Figure 1). Consequently the treatments are referred to as “lake-like” for limnetic prey or “stream-like” for benthic prey. For the lake-like treatment, live zooplankton (mainly *Daphnia sp.* and limnetic copepods), collected from Lake Lucerne, Switzerland using a 170 µm zooplankton net, was provided every day. For the stream-like treatment, live bloodworms (*Chironomidae* spp. larvae) were provided daily. To require a more realistic benthic feeding behaviour from the fish, bloodworms were introduced through a plastic tube separating them from the fish and allowing them to attach to the substrate. The plastic tube was then removed after five minutes. Fish were fed once per day until week 23 after hatching. Individuals were not fed for 24 hours before the end of the experiment. After the experiment, all individuals were sacrificed with an overdose of anaesthetic MS-222, weighed to the nearest 0.01g and preserved in 95% ethanol.

Ethics

All necessary permits were obtained to sample sticklebacks for the described field studies from the St. Gallen cantonal fishery authorities. Fish husbandry followed the Swiss veterinary legislation in concordance with the federal veterinary office (FVO) and was approved by the cantonal office in Bern (Veterinärdienst des Kantons Bern).

Estimating growth through time

Family-based differences in body size over time were estimated by taking standardised pictures of all individuals per tank in a plastic container with a 1x1 mm grid on the bottom and a water level of 1.5 cm (Figure 3a). Pictures were taken every two weeks starting on the first treatment day. Standard length of each individual was measured using IMAGEJ 1.43i (Abràmoff *et al.*, 2004) using the grid on each picture as a reference. Individual size at the beginning of the experiment was compared between source populations and treatments using a linear mixed effect model with family as random factor. Relative growth rates, measured as the difference in size over time, were statistically compared between source populations within treatments using a repeated measurement ANOVA with families as random factor. Experimental week was treated as a numerical variable, which allowed the estimation of the overall trend over time. Comparisons across treatments were not performed except for the comparison at the beginning of the experiment up to which point all individuals should have

experienced a similar raising environment (i.e. *Artemia* nauplii and *Panagrellus*). All statistical analyses were performed in R 2.12.1 (The R Core Team 2012).

Results

Differentiation of wild fish

The percentage of planktonic prey found in stomachs differed significantly across sampling events (X_3 = 19.20, p < 0.001), being significantly higher in the lake population, sampled in March 2009 compared to both stream samplings (March: t = -4.09, p < 0.001; July: t = -8.99, p < 0.001) and the lake population sampled in July (t = -7.50, p < 0.001) (Figure 1a). However, the lake population sampled in July did not differ in the percentage of planktonic prey from the stream populations sampled in March (t = 0.02, p = 0.987) or July (t = -1.57, p = 0.121). Wild caught lake fish fed mainly on cladocerans in March with a relatively small fraction of chironomid larvae, whereas the stream fish feed mainly on chironomids (Figure 1b). In July individuals from both habitats fed predominantly on chironomids.

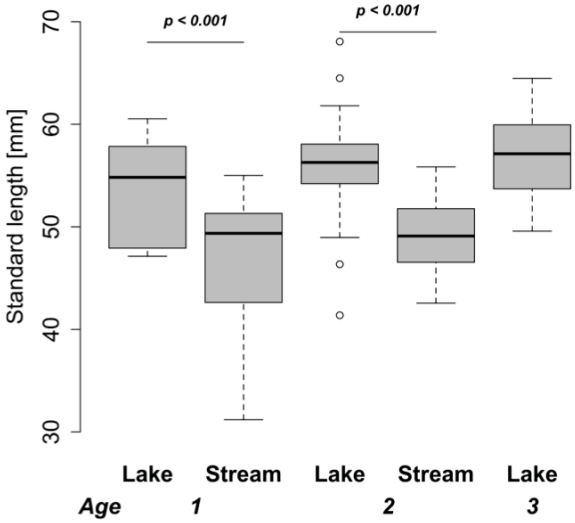

Figure 2: *Distribution of standard lengths for the lake and stream habitat from wild caught adult individuals for each age class. No three-year-old stream fish were obtained. Significant size differences between habitats within an age class, based on Tukey's HSD post hoc tests are indicated. Overall lake fish are significantly larger (p < 0.001) when accounted for age.*

The wild caught fish that were obtained together with the parents of the experimental individuals differed significantly in age between habitats, with lake fish being older than stream fish (average lake: 2.4 years, average stream: 1.7 years; $F_{1,138}$ = 44.07, p < 0.001). Size differed consistently between habitats for one and two year old individuals with lake fish being consistently larger (Figure 2), whereas size did not statistically differ between age classes within habitat (all p > 0.05). Overall, lake fish were significantly larger than stream fish when age was accounted for ($F_{1,137}$ =57.45, p < 0.001).

Experimental fish

In total, 441 out of 511 individuals survived until the end of the experiment (average overall mortality: 13.9% ± 16.1% SD). Mortality was highest for lake fish in the lake-like treatment (24.0% ± 26.1% SD) and lowest for stream fish in the lake-like treatment (4.1% ± 3.3% SD), whereas mortality was relatively similar in the stream-like treatment (lake fish: 12.6% ± 9.2% SD; stream fish: 14.2% ± 9.2% SD). Mortality was however not statistically different between treatments ($F_{1,22}$ = 0.04, p = 0.849) or source populations ($F_{1,22}$ = 2.54, p = 0.126) with a non significant interaction ($F_{1,22}$ = 3.46, p = 0.076) between them.

Although individuals were randomly assigned to each treatment, standard length differed between treatment groups five weeks after hatching at the beginning of the experiment, with individuals in the stream-like treatment being significantly larger ($F_{1,495}$ = 18.85, p < 0.001). Source populations on the other hand did not differ ($F_{1,11}$ = 0.10, p = 0.754), and the interaction of source and treatment was not significant ($F_{1,495}$ = 0.36, p = 0.548).

Size differed significantly over time between the lake and the stream population in the lake-like treatment ($F_{1,2382}$ = 9.66, p = 0.002) with lake fish growing larger than stream fish (Figure 3b). In the stream-like treatment, populations did not differ significantly in body size over time ($F_{1,2299}$ = 2.03, p = 0.155, figure 3c). For both stream and lake populations, individuals in the lake-like treatment grew faster than those in the stream-like treatment (stream: $F_{2,2235}$ = 10.44, p = 0.001; lake: $F_{2,2457}$ = 10.97, p < 0.001). At the end of the experiment, fish from the lake-like treatments (regardless of source population) were slightly longer ($F_{1,427}$ = 12.06, p < 0.001), but did not differ in body weight ($F_{1,427}$ = 0.05, p = 0.810) compared to fish from the stream-like treatments. Experimental fish did not differ between source populations at the end of the experiment (length: $F_{1,11}$ = 0.84, p = 0.381; weight: $F_{1,11}$ = 2.02, p = 0.183) with the interaction between source population and treatment being not significant for both length ($F_{1,426}$ = 0.39, p = 0.531) and weight ($F_{1,426}$ = 0.36, p = 0.549).

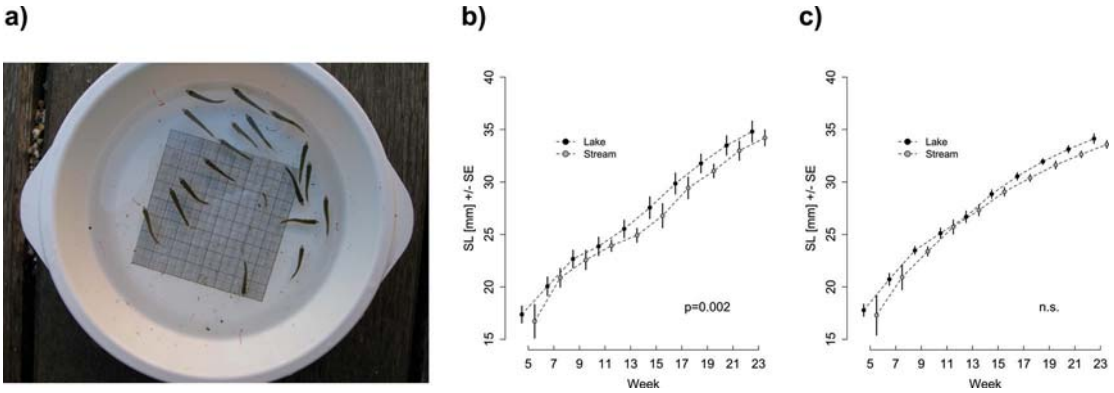

Figure 3: *a) Illustration of the method used to estimate average family-based body size over time. A 1x1 mm grid was attached to the bottom of a standardized plastic container, where the water level was kept at 1.5 cm. Panels b and c show the average body size over time for lake and stream populations under either b) lake-like or c) stream-like food treatment. Dots represent the mean standard length (SL) of all families per source population (± 1 SE).*

Discussion

In this study, we experimentally tested for a genetically determined evolutionary diversification during a biological invasion in a species known to occasionally form ecotype pairs within its natural range (Day *et al.*, 1994; Schluter, 1995; Hendry *et al.*, 2002; Sharpe *et al.*, 2008). We find that in the lake-like food treatment lake fish grow faster than stream fish. In the stream-like food treatment on the other hand, we find no significant difference between individuals from the two populations in their growth. These results provide experimental indications for putatively adaptive diversification, associated with the exploitation of different ecological niches can occur during a biological invasion. This has otherwise been shown only in few cases (Keller & Taylor, 2008), where adaptation and diversification have mostly been only indirectly inferred (e.g. Mathys & Lockwood, 2011; Le Rouzic *et al.*, 2011). However, phenotypic diversification in newly colonised habitats is a common phenomenon in invasive species (Bell *et al.*, 2004; Kristjánsson, 2005; Vellend *et al.*, 2007; Keller & Taylor, 2008; Wund *et al.*, 2008). Given that it provides the possibility to express advantageous phenotypes readily in a broad range of environments, phenotypic plasticity has often been invoked to explain phenotypic divergence in general (Ghalambor *et al.*, 2007) and for stickleback in particular (Wund *et al.*, 2008). In contrast, we found indications for a genetically determined fitness component separating the two ecotypes after less than 140 years since introduction in one comparison. Such a genetic basis could derive from multiple introduction events where different genetic lineages could admix. This could then lead to an increase of the adaptive genetic potential in the admixed population upon which divergent selection can act (Lavergne & Molofsky, 2007). Alternatively *in situ* evolution potentially based on ancestral standing genetic variation may account for the observed diversification. Because both populations originate from the same genetic lineage (Lucek *et al.*, 2010), diversification has likely occurred *in situ*. However, we are not able to determine if the lake population evolved from the stream population or *vice versa* through divergent adaptation. The first scenario seems to be more likely as sticklebacks were historically first observed in a stream close to our stream site in 1870 (Lucek *et al.*, 2010).

Niche expansion during invasion, i.e. the colonisation of divergent habitats, together with an increase in the diversity of utilised resources, may be attributed to ecologically driven diversification. This could represent a first step towards adaptive diversification (Yoder *et al.*, 2010), where heritable specialisation characterizes the second step along the invasion-diversification continuum (Keller & Taylor, 2008; Yoder *et al.*, 2010). Fitness trade offs between populations may arise if ecotypic specialisation for different resources occurs as a result of divergent natural selection (Schluter, 1995). Further selection could then lead to the fixation of alternative phenotypes with their underlying genotypes between ecologically differentiated populations, ultimately leading to ecological speciation (Nosil, 2012). Similarly, rapid phenotypic differentiation and diversification in sticklebacks, especially in body shape and defense related phenotypes has been shown to occur repeatedly along the marine – freshwater transition (Bell *et al.*, 2004; Kristjánsson, 2005; Wund *et al.*, 2008). Here, the rapid differentiation in lateral plate number has been attributed to selection on

standing genetic variation (Barrett et al. 2008; Le Rouzic *et al.*, 2011). Experimental assessments for a genetic differentiation in feeding related phenotypic traits have however only rarely been conducted, which suggest a mainly plastic contribution (Wund *et al.*, 2008).

Our finding that lake fish are able to utilise limnetic prey better than stream fish compared to benthic prey where lake fish grow at similar rates as stream fish indirectly suggests adaptive diversification along a parapatric benthic-limnetic axis. This is consistent with ecotype formation of sticklebacks in their natural range, where similar ecotypes as the ones observed here usually evolved over millennia and where divergence therefore is likely to be much older than in our study system (Day *et al.*, 1994; Hendry *et al.*, 2002; Sharpe *et al.*, 2008). In these systems, consistent adaptive divergence was found for both sympatric ecotypes (Day *et al.*, 1994), whereas a reciprocal transplant experiment between parapatric lake and stream populations showed different responses in each tested environment (Hendry *et al.*, 2002). In the later case, lake dwelling fish grew faster than stream dwelling ones in a lake environment, whereas both grew similarly in the stream environment. Using a controlled laboratory experiment, we obtain a similar pattern. The difference between these sympatric and parapatric comparisons may arise through different strengths of divergent selection, i.e. where intraspecific competition may increase divergent selection or cause disruptive selection in sympatry but not in parapatry (Bolnick & Lau, 2008).

In concordance with the abovementioned experiments in the wild, our experiment suggests that lake fish are able to grow on stream-like food at the same rate as stream fish, and may intrinsically grow faster and bigger. Although size differed only marginally between source populations within one of our experimental treatments at any point in time, the repeated measurement ANOVA supports a significant difference in body size through time in the lake-like treatment feeding on limnetic prey, suggesting a different growth rate between the ecotypes. This observed growth difference could result in different adult sizes, which is consistent with the size differences observed in the wild, where lake fish are significantly larger even when corrected for their age (Figure 2).

The absence of differentiation in the stream-like treatment could may further suggest different levels of local adaptation between the two tested populations (Kawecki & Ebert, 2004). Therefore behavioural versatility may be maintained, which would allow lake fish to switch more readily between the different feeding regimes (Wund *et al.*, 2008). Such behavioural versatility can be beneficial in heterogeneous environments where individuals encounter different feeding regimes. This may be the case in our system where lake fish feed on plankton in the open lake outside the breeding season, but enter shallow inshore waters, such as stream mouths for breeding. Here they start to increase feeding on benthic food, which is the most common locally available prey type. Stream fish on the other hand forage in streams throughout the year where they predominantly feed on locally available benthic prey (Figure 1). Consequently specialization may be reduced in the lake population as a consequence of the more heterogeneous feeding environment in comparison to the stream population, which feeds predominantly on benthic macroinvertebrates

throughout the year. However, our experimental setup using F1 offspring from wild parents does not allow to exclude potential maternal effects, which could be responsible for the higher intrinsic growth rate in stream fish. Indeed the parental populations used in our experiment differed in both their average age and their body size, which suggests a different life history strategy (Baker *et al.*, 2005). Such maternal effects could be either environmentally or genetically determined and hence be adaptive as well (Baker *et al.*, 2005), but further experiments are needed to estimate the contributions of maternal effects.

The observed pattern between the wild populations, where size differs between habitats in all age classes but age classes do not differ within habitats suggests that divergence in growth rates occurs mainly during the first year of life. Such size difference could be caused by selection due to different predation pressures in the two habitats, since increased body size could facilitate escape from gape-limited predators (Reimchen 1994). Indeed, experimental evidence suggests that sticklebacks are able to increase their growth rate as a plastic response to the presence of a predator, where larger individuals escape gape limited predators (Frommen *et al.*, 2011). In contrast, our experimental individuals were not exposed to predators, suggesting a genetic basis for increased growth rather than plasticity. Furthermore, even if predation is a main driver for the observed divergence in growth rates in the wild, further adaptations are needed to feed on zooplankton, i.e. forming limnetic feeding type phenotypes in sticklebacks (Wund *et al.*, 2008). Although divergence in specific feeding related phenotypes has been shown before in our system (Berner *et al.*, 2010) especially in gill raker length (Lucek *et al*., 2013), our experiment did not allow to investigate the relative growth trajectories of these traits as they either require increased handling or the individual to be sacrificed (e.g. gill rakers) (Wund *et al.*, 2012).

The evolutionary diversification that we observe in our study system may have further implications for both the present native species and the ecosystem itself. By exploiting different niches, sticklebacks are likely to introduce divergent selection pressure through interspecific competition and divergent predation pressure on their prey (Vellend *et al.*, 2007). Moreover, it has been shown that in their native range divergent stickleback ecotypes can each affect the community composition of lower trophic levels in different ways (Harmon *et al.*, 2009). This can further change the trophic interactions of other species. Indeed, experimental evidence suggests that lake dwelling sticklebacks from Lake Constance exert strong predation pressure on important herbivorous macroinvertebrates (Miler *et al.*, 2008). Here, stickleback predation changes both population size and growth of the prey by altering the sex ratios of the herbivores, which could then affect the ecosystem by increasing the vegetation density (Miler *et al.*, 2008). Consequently, invasive sticklebacks (Lucek *et al.*, 2010; Adachi *et al.*, 2012) might serve as a model system to further study evolutionary aspects and consequences of species invasion.

Acknowledgment

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Chapter 5

Disentangling the role of phenotypic plasticity and genetic divergence in contemporary ecotype formation during a biological invasion

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Abstract

The occurrence of contemporary ecotype formation through adaptive divergence of populations within the range of an invasive species typically requires standing genetic variation but can be facilitated by phenotypic plasticity. Yet, the relative contributions of both of these to adaptive trait difference have rarely been simultaneously analyzed in recently colonized systems, especially for vertebrates. Here we study a case of intraspecific divergence into distinct lake and stream ecotypes of threespine stickleback that evolved in the past 140 years within the invasive range in Switzerland. Using a controlled laboratory experiment with full-sib crosses and treatments mimicking a key feature of ecotypic niche divergence, we test if the phenotypic divergence that we observe in the wild results from phenotypic plasticity or divergent genetic predisposition. Our experimental groups show qualitatively similar phenotypic divergence as those observed among wild adults. The relative contribution of plasticity and divergent genetic predisposition differs among the traits studied, with traits related to the biomechanics of feeding showing a stronger genetic predisposition, whereas traits related to locomotion are mainly plastic. These results implicate that phenotypic plasticity and standing genetic variation interacted during contemporary ecotype formation in this case.

Introduction

Contemporary phenotypic evolution associated with adaptation to ecologically contrasting environments is a common phenomenon especially during biological invasions, i.e. the establishment and spread of a species in a non-native environment. This has been studied in plants (Bossdorf *et al.*, 2005; Colautti *et al.*, 2010; Calsbeek *et al.*, 2011), invertebrates (Huey *et al.*, 2000; Carroll *et al.*, 2001; Lee *et al.*, 2003) and vertebrates (Reznick & Endler, 1982; Hendry *et al.*, 2000; Koskinen *et al.*, 2002), and phenotypic change is consistently associated with the invasion process (Elton, 1958; Baker, 1980; Herrel *et al.*, 2008; Keller & Taylor, 2008). However, ecotype formation, the formation of ecologically and phenotypically differentiated populations that occupy different environments within the invaded range, has less often been described (e.g. Hendry *et al.*, 2000; Carroll *et al.*, 2001; Koskinen *et al.*, 2002; Phillips & Shine, 2006; Calsbeek *et al.*, 2011). Although contemporary phenotypic evolution may represent a common feature in biological invasions (see Reznick & Ghalambor, 2001; Carroll *et al.*, 2007; Westley, 2011), the respective roles of genetic determination, phenotypic plasticity or their interplay in promoting or impeding such rapid adaptive responses is still debated.

Depending on the amount of gene flow between habitats with different requirements for adaptation, phenotypic divergence can evolve fast if standing genetic variation in relevant genes permits the emergence of beneficial phenotypes and their exposure to selection (Facon *et al.*, 2006; Barrett & Schluter, 2008; Lee *et al.*, 2011). Sorting of the preexisting alleles can then lead rapidly to adaptive and heritable phenotypic differentiation between populations (Nosil, 2012). In contrast, genetically depauperate populations would need time for advantageous genetic variation to arise, a process thought to be partly responsible for the so-called "lag phase" in biotic invasions (Sakai *et al.*, 2001).

Beneficial phenotypes may also be expressed through phenotypic plasticity, where ancestral genotypes would be able to express different phenotypes in different environments (Price *et al.*, 2003; Pfennig *et al.*, 2010). Such divergent trait expression between habitats can itself become genetically fixed over time either through phenotypic canalization combined with genetic assimilation (Weinig, 2000; Yeh & Price, 2004; Crispo, 2007; Lande, 2009; Thibert-Plante & Hendry, 2011) or genetic accommodation, where selection acts on the reaction norm itself but may retain some plasticity (West-Eberhard, 2003). In both cases, successful genetic change depends among other factors, including the strength of selection, the costs of maintaining plasticity, and the stability of the selection regime (West-Eberhard, 2003; Crispo, 2008; Lande, 2009; Thibert-Plante & Hendry, 2011). Plasticity may also shield the genome from the effects of selection and hence prevent a genetic fixation (Price *et al.*, 2003; Ghalambor *et al.*, 2007). The effect of plasticity on the strength of divergent selection further depends on timing. If plasticity is expressed early in ontogeny before possible dispersal between contrasting habitats, divergent selection can be strong because selection against immigrants can occur, whereas expression after dispersal may dissipate divergent selection (Thibert-Plante & Hendry, 2011).

By raising wild populations with experimental treatments that mimic a key feature of habitat contrasts between ecotypes, we can experimentally test for the relative roles of plasticity and genetic determination in contemporary phenotypic evolution (Kawecki & Ebert, 2004). Though this can only be achieved in recently diverged biological systems, such as in recent biological invasions (Carroll *et al.*, 2007; Lande, 2009). Yet, the contributions of both plasticity and genetic determination underlying adaptive trait divergence have rarely been simultaneously distinguished as few studies have used a combination of common rearing environments and environmental manipulations in recently evolved systems, especially in vertebrates (e.g. Robinson & Wilson, 1996; Weinig, 2000; Carroll *et al.*, 2001; Lee & Petersen, 2002; Lee *et al.*, 2003; Colautti *et al.*, 2010; Collyer *et al.*, 2011, see Ghalambor *et al.*, 2007 for a review). Disentangling the relative effects of genetics and plasticity is important because biological invasions that lead to the formation of distinct ecotypes can sometimes lead to ecologically differentiated species (Adams & Huntingford, 2004) and even to adaptive radiations (Simpson, 1953; Schluter, 2000; Yoder *et al.*, 2010).

The threespine stickleback (*Gasterosteus aculeatus* species complex) recurrently colonized freshwater environments from ancestral marine populations throughout its Holarctic distribution. In freshwater they repeatedly radiated into different habitat specialists, forming genetically distinct ecotypes and species (McKinnon & Rundle, 2002; Hendry *et al.*, 2009). This ecotypic differentiation can be partially attributed to adaptive phenotypic plasticity (Day *et al.*, 1994) that is already present in their marine ancestors (Wund *et al.*, 2008). In many cases, the observed ecological differentiation among these taxa is manifested in functionally relevant changes, e.g. mouth shapes adapted in stream habitats to suction feeding on invertebrates attached to the substrate in streams, compared to more ram feeding on zooplankton in lake habitats (Caldecutt & Adams, 1998). Whereas many natural population pairs began diverging shortly after the last glaciation ~15'000 years ago, sticklebacks arrived in the midlands of Switzerland only ~140 years ago (Lucek *et al.*, 2010). Since then, they underwent a massive range and niche expansion, now occupying habitats as different as very large oligotrophic lakes, rivers, ponds and small streams. Coinciding with this, repeated phenotypic divergence occurred. Of particular interest is the divergence between physically connected lake and stream habitats because such divergence in the absence of strong geographical isolation can inform us about evolutionary mechanisms and constraints. Invasive sticklebacks in Switzerland formed lake-stream pairs similar to those found within the native range (Reimchen *et al.*, 1985; Hendry *et al.*, 2002; Berner *et al.*, 2009; Kaeuffer *et al.*, 2012; Ravinet *et al.*, 2013b) that differ especially in feeding-related morphology (Berner *et al.*, 2010; Roy *et al.*, 2010; Lucek *et al*. 2013). One of the most strongly divergent of the known Swiss lake-stream population pairs occurs in the Lake Constance region, where stomach content data (Lucek *et al.*, 2012a) and stable isotopic signature (Lucek et al. 2013) as well as morphological and life history data suggest divergence into distinct ecotypes (Lucek et al., 2012a; Moser *et al.*, 2012). This divergent ecotype pair originates from a single colonization event (Lucek *et al.*, 2010) and is weakly genetically differentiated at neutral markers, but occasional gene flow may still occur (Lucek et al. 2013).

To test if the observed and potentially adaptive phenotypic differentiation of this ecotype pair results from environmentally induced adaptive plasticity or from divergent genetic predispositions, we used a controlled laboratory experiment (Robinson & Wilson, 1996; Adams & Huntingford, 2004; Proulx & Magnan, 2004; Sharpe *et al.*, 2008). We raised full-sib F1 families from each ecotype under two food regimes, mimicking the main prey categories found in the wild (Lucek et al., 2012a). Because invasion and establishment of sticklebacks in our study system occurred only within the last 140 years (Lucek *et al.*, 2010), we expected plasticity to be a main driver of phenotypic divergence. Thus we predicted that plastic traits would either differ according to food treatment or would not differ at all, whereas genetic predisposition should result in morphological differences between source populations independent of the food treatment. In both cases, we expected to observe phenotypic differences in feeding related functionally relevant traits that would resemble the differences seen in the wild between the two ecotypes and hence indicate for each trait the underlying nature of adaptive trait variation, plasticity or heritable variation, related to ecological divergence in the wild.

Material and methods

Fish collection and crossing

Ripe adult individuals from Lake Constance, Switzerland (47°29'02''N, 9°33'35''E) and a parapatric stream site (47°19'33''N, 9°34'41''E) were sampled in May 2010. Seven randomly selected pairs of lake males and lake females, and six randomly selected pairs of stream males and stream females were placed in individual 60 x 30 x 40 cm aquaria (one pair per tank). Each tank was equipped with sand substrate, natural nesting material as well as a filtering and aerating system. Following a successful spawning event, both adult fish were removed and sacrificed with an overdose of anesthetic MS-222 and preserved in ethanol. A random population sample of wild adult fish was taken at the same time when collecting the parental fish (N_{lake} = 96, N_{stream} = 49) in order to obtain the phenotypic distributions from which the parents were drawn. The same was done in October 2010, at the end of the experiment, to obtain the phenotypic distributions of wild young of the year (YOY) individuals (N_{lake} = 40, N_{stream} = 44).

Husbandry and experimental setup

Fertilized eggs were separately aerated in each tank. Eggs with fungal infection or dead embryos were removed daily. Two thirds of the water in each tank was replaced every two days throughout the experiment. All hatched individuals were fed with *Artemia sp.* nauplii for the first five weeks after hatching. Between weeks four and five, small nematodes (*Panagrellus sp.*) were also provided. After week five, each full-sib family was split into two halves of 18-20 individuals each, experiencing from week six onwards either a "plankton" type or a "benthos" type food regime. For the plankton treatment, live

zooplankton (mainly *Daphnia sp.* and limnetic copepods), collected with a 170 µm zooplankton net from Lake Lucerne, Switzerland, was provided as food every day. For the benthos treatment, live bloodworms (*Chironomidae* spp. larvae) were provided daily. These food items are similar to the main prey items eaten in the wild (Lucek et al., 2012a). To furthermore require a more realistic benthic type feeding behavior from the fish, bloodworms were introduced through a plastic tube separating them from the fish and allowing them to attach to the substrate. The plastic tube was then removed after five minutes, allowing the fish to feed by picking bloodworms out from the substrate. Fish were fed once per day till week 23 after hatching. After the experiment, all individuals were sacrificed with an overdose of anaesthetic MS-222 and preserved in 95% ethanol. In order to highlight bony structures, all individuals were stained using a protocol from Peichel et al. (2001), followed by a bleaching step with a solution of 0.6% KOH and 1.2% H_2O_2.

Morphological analysis

To quantify relevant phenotypic differentiation among groups, a set of morphological traits known to be often divergent among stickleback ecotypes were measured (Day *et al.*, 1994; Berner *et al.*, 2008): standard length, body depth, head length, head depth, eye diameter, upper and lower jaw length, snout length and gape width. Standardized pictures were obtained from the left side of each stained fish with a flat-bed scanner on which all linear measurements were then taken using IMAGEJ 1.43u (Abràmoff *et al.*, 2004), except for gape width. The latter was measured as the ventral distance between the posterior-most points of premaxillary bones of each side to the nearest 0.01 mm using a digital caliper. In addition, the number of gill rakers, the gill arch length and the length of the second gill raker, as counted from the joint of the dorsal arch bone and measured from its tip to the insertion on the gill arch, were determined using a dissection microscope with a micrometer attached. Because all measurements except gill raker numbers were significantly correlated with size (results not shown), a size correction was applied by taking the residuals of a regression of each untransformed linear trait against standard length. This was performed separately for the experimental individuals, wild caught adults and YOY using each a single within-group regression to account for allometric differences between these groups.

Overall multivariate differentiation among experimental fish was tested in three ways: First a linear discriminant (LD) analysis was performed using all linear traits with food treatment and source population separately as grouping variables to identify trait contributions associated with either response variable. The classification success, which is defined as the average probability among individuals for each group to be assigned to their own group, was then extracted from the LD model. In addition, the degree of differentiation between groups was estimated as their pairwise Mahalanobis distances. Second, an analysis of multivariate variance (MANOVA) was performed with family as a random factor to test for an overall statistical phenotypic differentiation between either food

treatment or source population. Third, all linear traits were summarized using a PC analysis, retaining the scores for each individual for the three leading PC axes.

Each trait and PC axis was analyzed using a mixed linear model including *food treatment* (plankton or benthos) and *source population* (lake or stream) as explanatory variables and family as a random factor. The significance levels of the explanatory variables were assessed using a backward elimination procedure based on type II *F* tests (see Lemoine *et al.*, 2012 for details). The effect size of *food treatment*, *source population* and the *food treatment* x *source population* interaction was further estimated using Cohen's D in order to quantify the relative contributions of plasticity and genetic predisposition.

Differentiation between habitats was similarly tested for the wild caught populations separately for adults and YOY using a LD analysis and a MANOVA. Likewise, the classification success and the degree of differentiation measured as pairwise Mahalanobis distances were calculated. In addition, each measured morphological trait was separately compared between wild caught populations using *t*-tests.

Shape analysis

Geometric morphometrics was used to capture shape variation in wild caught and experimental fish. Nineteen landmarks were selected that cover overall body shape with an emphasis on head shape and traits related to functional morphology of the feeding apparatus (Anker, 1974; Walker, 1997; Caldecutt & Adams, 1998) Table S1). All landmarks were placed on dyed bone structures. Landmarks were set using TPSDIG2 (Rohlf, 2006), with individuals in random order. Procrustes fits were performed on the obtained data sets separately for wild adults, wild YOY and experimental fish in MORPHOJ 1.03b (Klingenberg, 2011). Procrustes coordinates were size corrected by a regression against standard length retaining the residuals. A canonical variate (CV) analysis on these residuals was performed, based on pooled within group covariances to identify the multivariate axis that explains most variation between groups. Groups represented either source population (lake and stream) for both experimental and wild caught fish or food treatment for experimental fish only. For each analysis, the classification success for each group was extracted from MORPHOJ (Klingenberg, 2011). Furthermore the degree of differentiation among groups was estimated as pairwise Mahalanobis distances, whose significances were estimated using a bootstrap approach with 10000 replicates implemented in MORPHOJ. In addition, a PC analysis was conducted retaining the scores of the three leading PC axes, where significances among groups were similarly calculated as for the linear measurements. To further illustrate the phenotypic changes associated with differences in CV scores, deformation wire frame graphs were 2.5 times exaggerated (Wund *et al.*, 2008).

Trait loadings along each CV axis for shape data and along each LD axis for linear morphology were standardized for each axis separately by dividing the absolute trait loadings with the highest observed loading on each axis. In order to compare the observed differentiation along the LD or CV axes for experimental individuals in relation to their wild type counterparts, the latter were furthermore projected into the morphospace of the experimental

individuals using the package MASS (Venables & Ripley, 2002) in R 2.15.1 (R Core Team, 2012). In short, this projection approach uses the loading vectors of each LD or CV that separate either source populations or treatments for experimental individuals and calculates the residuals for each projected individual along each given axis. These projected scores can then be subsequently analyzed. Similarly both the experimental individuals and wild caught YOY were projected onto the multivariate axis that separates the wild caught adult populations. Finally, the residuals of each LD, PC and CV analysis were subsequently regressed against standard length, to test if the multivariate differentiation was driven by allometric information that might have been retained after the size correction. Similarly, MANOVAs were performed for linear measurements using size as a factor.

Finally to test if the parental individuals used for the experimental crosses represent a random subset of the phenotypic distribution in the wild, they were first projected onto either the LD axis separating the wild caught populations for liner measurements or the CV axis for body shape. The obtained individual scores were then statistically compared between parents and wild caught individuals separately for the lake and stream population using *t*-tests.

Results

In total, 441 individuals were alive at the end of the experiment. Overall mortality was 13.9% ± 3.2 SE, and did not statistically differ between *food treatment* ($F_{1,22}$ = 0.04, p = 0.849) or *source population* ($F_{1,22}$ = 2.54, p = 0.126) with a non significant interaction ($F_{1,22}$ = 3.46, p = 0.076) between them. Fish tended to have higher mortality in their native treatment, but none of the pairwise comparisons were significant (all p > 0.100). Individuals in the plankton treatment were slightly but significantly larger than in the benthos treatment at the end of the experiment ($F_{1,426}$ = 3.91, p = 0.049), but size did not differ between source populations ($F_{1,11}$ = 1.31, p = 0.278) with a non significant interaction ($F_{1,426}$ = 0.39, p = 0.531).

Linear morphology - wild fish

Using size corrected linear morphology, wild caught adult fish differed between the stream and lake environment (MANOVA: $F_{1,139}$ = 4.07, p < 0.001, Mahalanobis distance: 1.167), which was similarly recovered with the LD analysis (Fig 1 & S1, Table S2). The classification success differed between the lake (lake fish assigned to the lake population: 70.8%) and the stream population (stream fish assigned to the stream population: 48.4%). In contrast, young of the year (YOY) individuals were not significantly differentiated in the overall multivariate analysis (MANOVA: $F_{1,82}$ = 1.01, p = 0.734, Mahalanobis distance: 0.734). This was similarly reflected with the LD analysis, where the classification success was similar for the lake (lake fish assigned to the lake population: 48.5%) and the stream population (stream fish assigned to the stream population: 51.1%). However, YOY showed significant differentiation in several traits between both environments (Table S3).

Wild adult lake fish had shallower bodies ($t_{1,139}$ = -3.98, p < 0.001) and deeper heads ($t_{1,139}$ = 3.03, p < 0.003) and were significantly larger than wild adult stream fish ($t_{1,139}$ = 9.73, p < 0.001; Table S3). Gill raker number was not different ($t_{1,139}$ = -0.70, p = 0.487) but gill rakers were significantly longer in the lake population ($t_{1,139}$ = 3.98, p < 0.001). Wild caught YOY from the lake were significantly smaller in size than YOY stream fish ($t_{1,82}$ = -12.06, p < 0.001). Wild caught stream YOY had significantly larger heads ($t_{1,82}$ = -5.58, p < 0.001), eyes ($t_{1,82}$ = -8.15, p < 0.001), lower jaws ($t_{1,82}$ = -4.54, p < 0.001), longer gill rakers ($t_{1,82}$ = -3.29, p < 0.001) and wider gapes ($t_{1,82}$ = -8.72, p < 0.001, Table S3).

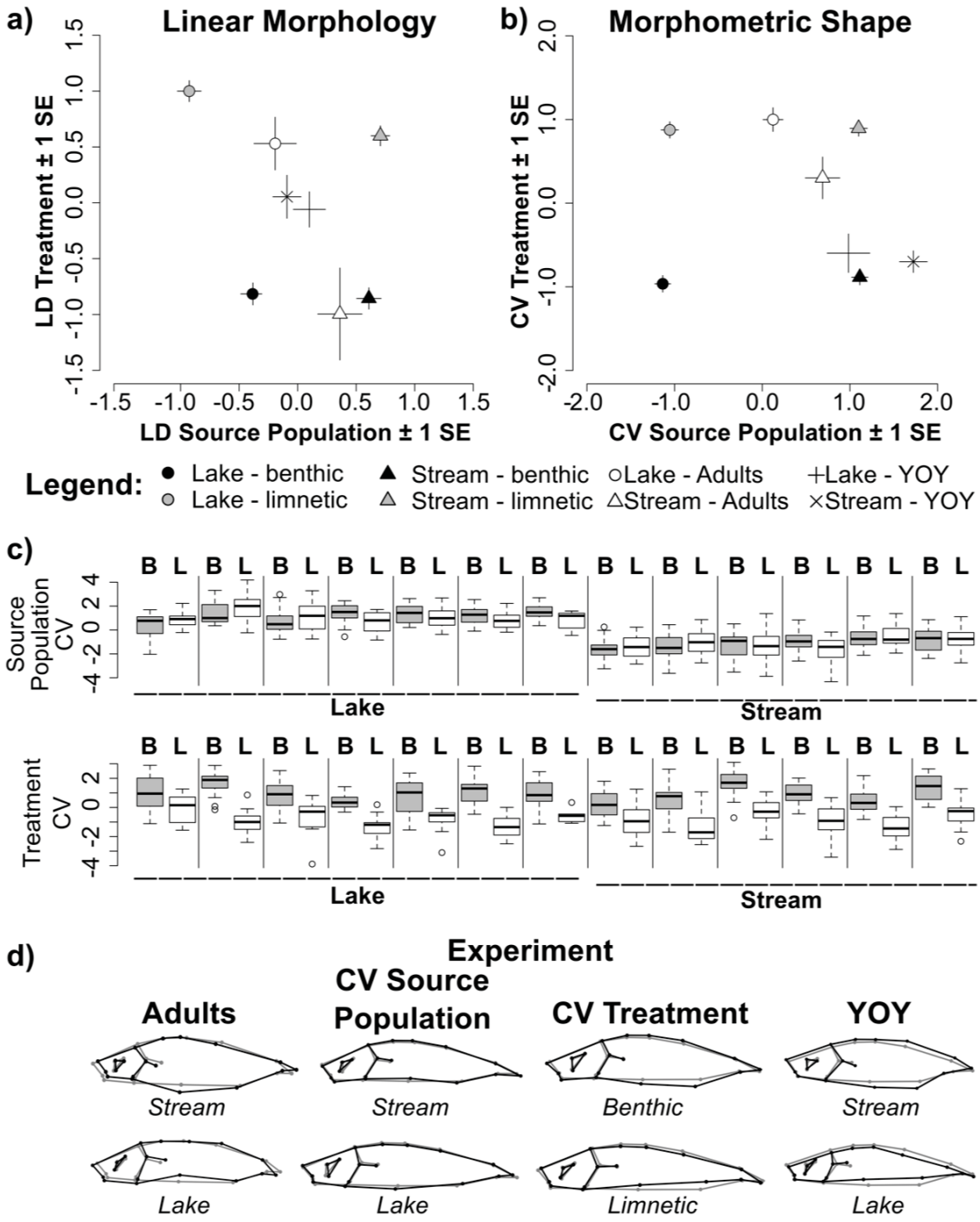

Figure 1: *Summary of the phenotypic changes observed for both linear morphology and morphometric shape. a) Average linear discriminant (LD) loadings on the leading axes calculated using either source population (lake, stream) or treatment (limnetic plankton, benthos) as grouping variable (± 1 SE). In addition, both wild caught young of the year (YOY) and adult individuals were projected into the morphospace of the experimental individuals (see main text for details). b) Average canonical variate (CV) loadings on the leading axes for source and treatment (± 1 SE) for all experimental fish with wild caught individuals being projected into the morphospace of the experimental individuals. c) Canonical variate (CV) scores separated for each family of the leading axis for morphometric shape data using either source population (lake or stream; top) or treatment (B - benthic or L – limnetic zooplankton; bottom) as grouping variable. d) Morphometric shape differences along the leading CV axis for wild caught adults, YOY and experimental individuals. Deformations (black) are 2.5x exaggerated to visualize the differences from the consensus (grey).*

Linear morphology - experimental full-sib F1 fish

For the experimental fish, the three leading principal component (PC) axes captured 75.2% of the total variation (48.9%, 16.0%, 10.3% on PC axis 1-3 respectively) with PC1 being significantly associated with food treatment (Fig 2, Table S4). PC2 and PC3 showed a significant *food treatment* x *source population* interaction and a significant *food treatment* factor, indicating different multivariate reaction norms of source populations (Fig S2). Effects of food treatment and source population differed among traits when each trait was separately analyzed (Fig 2, Table S4). Here, only gape width showed a significant interaction, indicating different reaction norms between source populations, with fish raised in their native like environment having wider gapes than in non-native like environment, where furthermore lake fish had wider gapes than stream fish in both food treatments (Fig S2). Significance levels were consistent with effect sizes; most linear traits had a high treatment-induced component, with the interaction showing the second strongest effect in most cases (Fig 2). Individuals from the benthos treatment had shorter heads with smaller eyes and deeper bodies (Fig S2). Some feeding related traits (gill raker length, gill arch length, lower jaw length) showed no significant difference in any comparisons (Fig 2, Table S4).

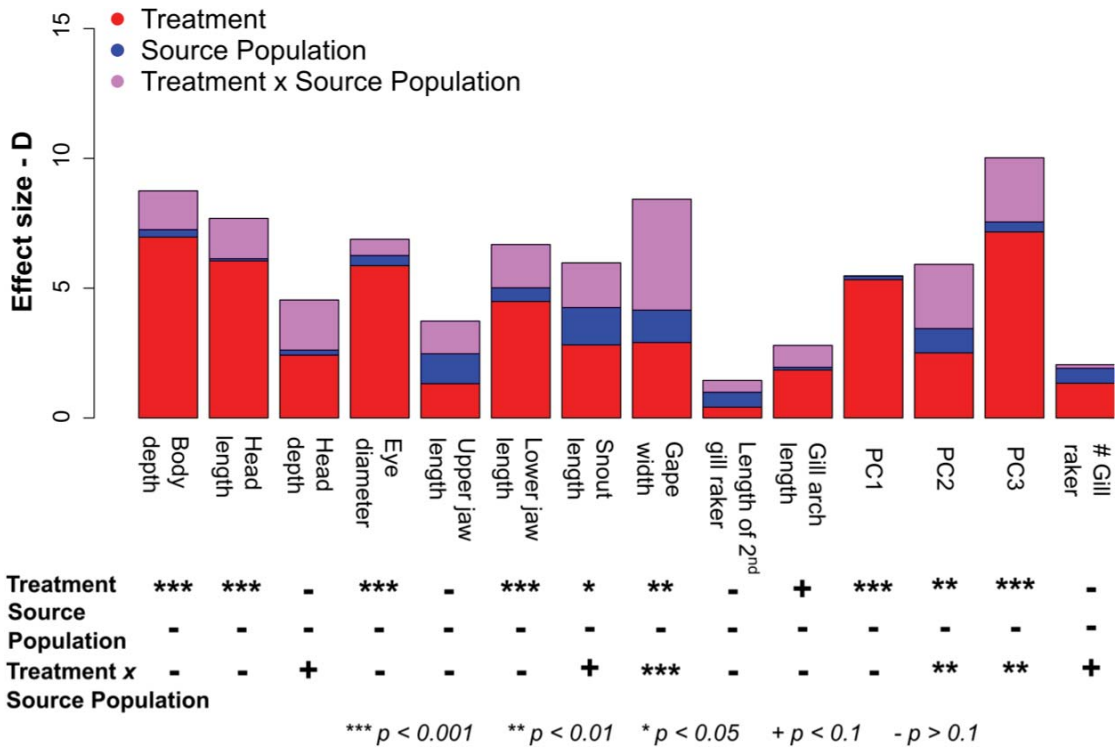

Figure 2: *Effect sizes (Cohen's D) for treatment, source and their interaction with the corresponding p values are given for all linear size-corrected morphological traits separately, and for these traits combined using a principal component analysis (PC; axes 1-3 indicated). Results for the number of gill raker are shown separately. The significance levels are based on mixed linear models with source population and treatment as response variable and family as random effect using a backward elimination procedure and a type II F tests (see Figure S2 and Table S4 for details).*

The multivariate analyses based on the linear measurements showed a significant separation between treatments (MANOVA: $F_{1,427}$ = 31.5, p < 0.001, Mahalanobis distance: 1.630) and source populations (MANOVA: $F_{1,427}$ = 18.6, p < 0.001, Mahalanobis distance: 1.312). This was similarly true for both LD analyses (Fig 1a), where gape width contributed highly on both axes (Fig S3, Table S2). The classification success was comparable among treatments (benthic fed fish assigned to the benthos treatment: 69.7%; limnetic fed fish assigned to the plankton treatment: 71.6%) and among source populations (lake fish assigned to the lake population: 64.7%; stream fish assigned to the stream population: 65.3%). When wild type individuals were projected into the morphospace of experimental individuals, YOY clustered closely together and were intermediate to the experimental individuals along both LD axes (Fig 1a). Wild caught adult individuals on the other hand clustered closely to their matching experimental counterpart. This was especially true for wild stream adults, resembling benthic-fed experimental stream fish (Fig 1a). However, when wild YOY and the experimental individuals were projected on the axis that separates wild caught adult populations (Fig 3), the experimental individuals segregate towards their matching ecotype.

Shape analysis – wild fish

Wild caught adults but not YOY, differed significantly in their multivariate shape between source populations with a similar decreased differentiation between source populations among YOY as observed with linear morphology (adults: Mahalanobis distance: 1.660, $p < 0.001$; YOY: Mahalanobis distance: 0.960 $p = 0.995$). This was similarly reflected by the classification success, which was higher for adults (average probability for lake fish being assigned to the lake population: 82.9%; stream fish to the stream population: 84.9%) than for YOY (lake fish to the lake population: 60.0%, stream fish to the stream population: 68.2%). Decreased differentiation among YOY was furthermore observed with the canonical variate (CV) analysis (Fig S1). Yet, both adult and YOY stream fish showed deeper bodies and smaller eyes (Fig 1). Head shapes differed among the wild caught adults, where lake fish had more elongated and deeper heads and longer jaws. In YOY, this was inversed with deeper heads in stream fish. Landmarks accounting for most of the phenotypic variation between wild populations for both adults and YOY were concentrated on the head (Table S5). These traits are furthermore mechanically important for the relative forces applied during feeding (Caldecutt & Adams, 1998).

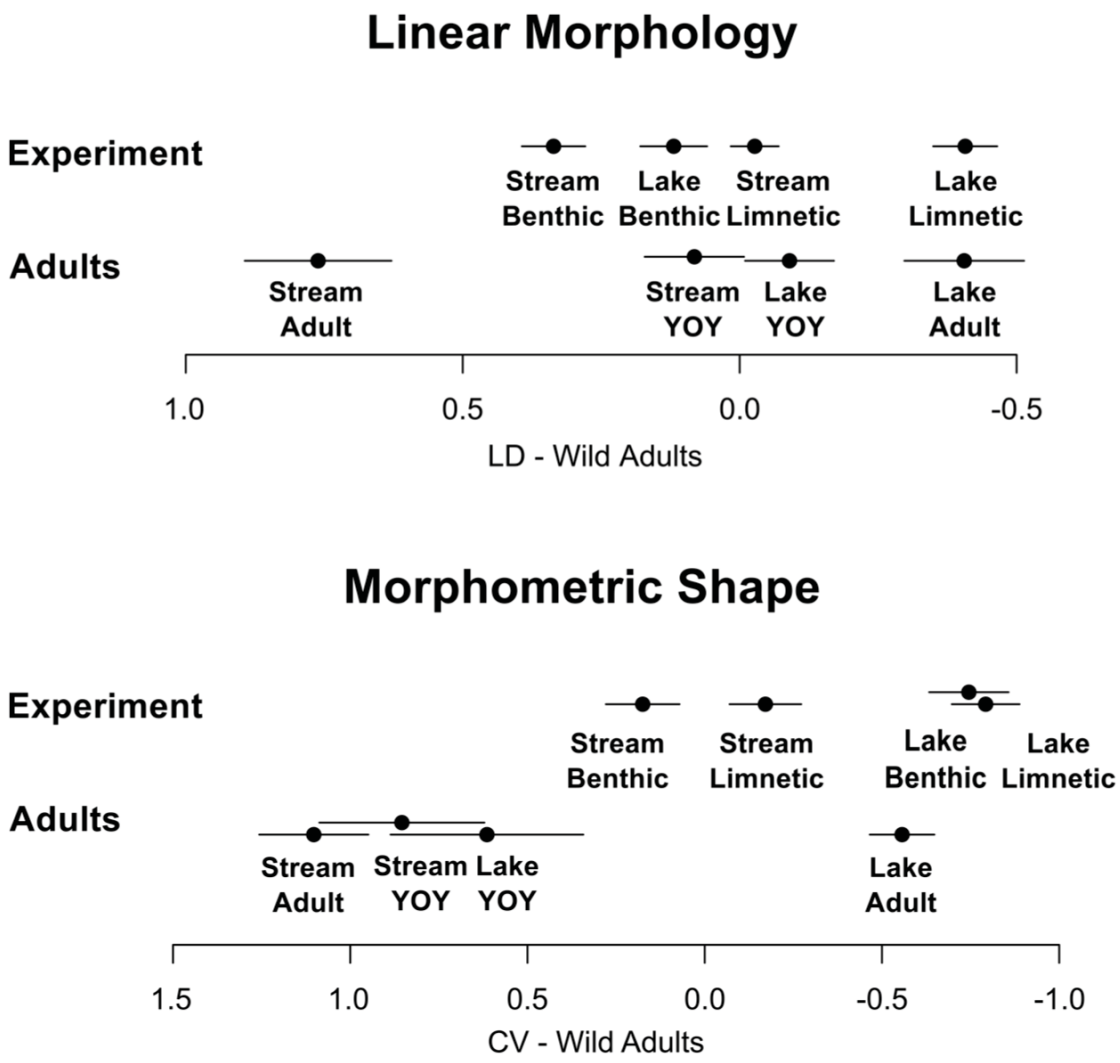

Figure 3: *Average linear discriminant (LD) scores (± 1 SE) for the axis separating both wild caught lake and stream individuals. In addition the average scores for the wild caught young of the year (YOY) individuals and the experimental groups are given, which are based on a projection onto the wild adult LD axis (see main text for details). LD axes are either base on linear morphology (top) or morphometric shape data (bottom).*

Shape analysis – experimental fish

The three leading PC axes on shape for experimental individuals captured 57.0% of the total variation (29.3%, 17.1%, 10.6% on PC axis 1-3 respectively). The shape changes captured by these PC axes were however mainly related to changes in the bending of a specimen and the vertical position of the tail, where only PC3 showed some changes in body depth (Fig S4). None of these axes showed a significant contribution of either *source, treatment* or their interaction except for PC3, where the best statistical model showed a significant treatment effect ($F_{1,426}$ = 4.0, p =0.047; Table S4). The CV axes for experimental fish on the other hand significantly separated both source populations (Mahalanobis distance: 2.201, $p < 0.001$) and food treatments (Mahalanobis distance: 1.812, $p < 0.001$; Fig 1 & 3). The relative classification success was comparable between the CV axes separating *treatment* (benthic fed fish assigned to the benthos treatment: 80.9%; limnetic fed fish assigned to the plankton treatment: 83.1%) and *source* (lake fish assigned to the lake population: 86.4%; stream fish assigned to the stream population: 84.9%). This differentiation was remarkably consistent among all 13 families within each analysis (Fig 1c). Traits that explained most variation on the CV axis, which separated experimental individuals according to their source population, involved changes along the anteroposterior axis, especially head shape: experimental individuals originating from the lake had a more terminal mouth with the maxilla dorsocaudally shifted and a shorter head (Table S5, Fig 1b & 3). Experimental stream fish showed a reduced orbit size, being linked to eye size with an increased suspensorium size (suspending the jaws from the neurocranium). The phenotypic differentiation for experimental individuals along the CV axis separating lake and stream originating individuals resulted in a similar phenotypic differentiation as observed between the wild caught adult populations, i.e. experimental lake fish had more elongated and deeper heads and longer jaws (Fig 1d).

On the CV axis separating food treatments, traits linked to head structure as well as body shape had higher loadings, which resulted in overall shape changes along the transversal body axis. Fish raised on benthic food had deeper bodies with a larger orbit and an increased suspensorium (Fig 1d). Individuals raised on plankton showed a more upturned snout with the premaxilla shifted along the anteroposterior axis although with low statistical support (Table S5). The comparison of standardized loadings of the two leading axes indicated five landmarks located on the head that differ mainly between source populations, being therefore likely genetically determined. Four other landmarks showed a relatively high treatment effect and therefore likely to be mainly driven by plasticity. These were linked to body depth (Fig 4). In addition, three traits, all

related to eye or orbit size showed an interaction between source population and the treatment, suggesting both genetic and plastic components.

When the wild caught populations were projected onto the CV axes that separate the experimental individuals, wild YOY from both the lake and the stream population clustered close to the experimental benthic fed stream individuals, whereas wild caught adults segregated towards the plankton treatment (Fig 1b). When the wild YOY and the experimental individuals were projected onto the CV axis that separates the two wild adult populations, wild YOY from both populations clustered similarly close to the wild adults from the stream site (Fig 3). Experimental fish that originate from the lake showed a more extreme phenotype than the wild caught adult lake fish. Experimental stream fish on the other hand fell phenotypically in between the wild caught adults of the two populations.

None of the statistical comparisons between the wild type adults and the individuals used to generate the experimental crosses were significant: LD scores of lake parents vs. wild type lake: $t_{1,105} = 0.07$, $p = 0.946$; LD scores of stream parents vs. wild type stream: $t_{1,60} = 1.38$, $p = 0.185$; CV scores of lake parents vs. wild type lake: $t_{1,104} = -0.88$, $p = 0.389$; CV scores of stream parents vs. wild type stream: $t_{1,52} = -0.98$, $p = 0.340$. This suggests that the parents used in the experiment represent a random subsample of each population. Finally, none of the estimated residuals from any multivariate analysis, i.e. LD, PC or CV, were statistically associated with standard length (all p = 1.000, results not shown). Similarly none of the MANOVA analyses that were performed using standard length as factor were statistically significant (all p > 0.900, results not shown). Consequently, none of the multivariate analyses were driven by size differences.

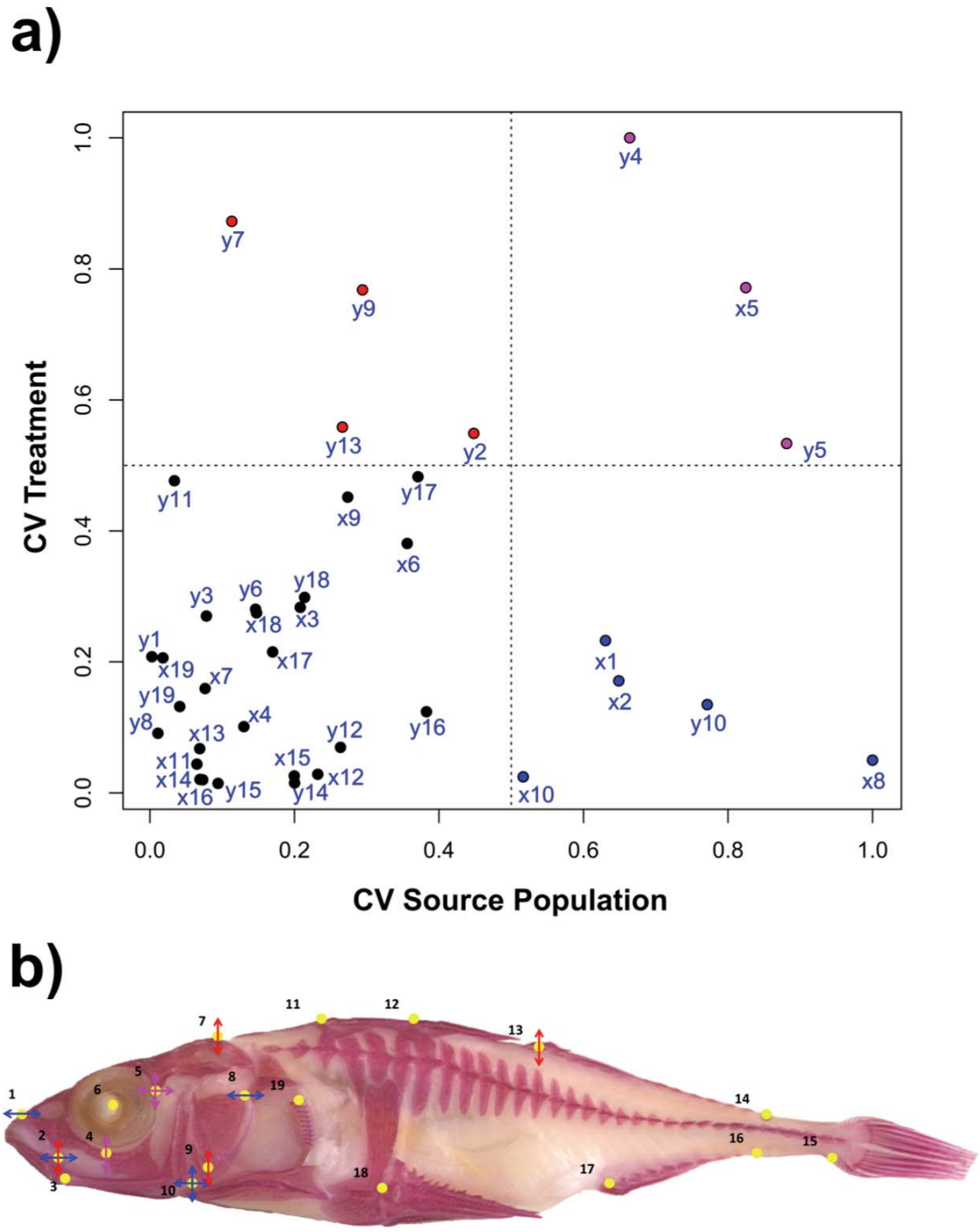

Figure 4: *Differentiating between plastic and genetically determined morphometric shape traits. a) Standardized relative trait loadings of the first canonical variate (CV) axes, calculated using either source population (lake, stream) or treatment (plankton, benthos) as grouping variable. Highlighted are traits showing high levels of genetic determination (above an arbitrary cutoff value of 0.5) and are hence either mainly genetically determined (blue), phenotypic plasticity (red) or by an interaction of both (pink). Each trait name consists of the spatial coordinates of its related landmark (i.e. x and y for changes along the horizontal or vertical axis respectively) and the landmark ID given in b. b) Landmark positions with arrows indicating the shape changes associated with the highlighted traits in a). See Table S1 for a detailed description of each landmark.*

Discussion

Evolutionary phenotypic divergence of invasive populations away from ancestral populations has commonly been found (e.g. Huey *et al.*, 2000; Lee *et al.*, 2003; Phillips & Shine, 2006; Carroll *et al.*, 2007; Keller & Taylor, 2008; Prentis *et al.*, 2008; Calsbeek *et al.*, 2011). Similarly, yet to a lesser extent, ecotype formation between distinct habitats within a recently invaded range has been demonstrated (e.g. Hendry *et al.*, 2000; Koskinen *et al.*, 2002; Phillips *et al.*, 2006; Keller & Taylor, 2008, see Keller & Taylor, 2008; Yoder *et al.*, 2010 for discussion). However, it is less clear if and to what extent such contemporary phenotypic evolution is triggered by either phenotypic plasticity (Weinig, 2000; Yeh & Price, 2004; Crispo, 2008; Lande, 2009; Thibert-Plante & Hendry, 2011) or selection on standing genetic variation (Facon *et al.*, 2006; Barrett & Schluter, 2008; Lee *et al.*, 2011). Cases of apparently rapid adaptive diversification from a single colonizing population are particularly interesting because they can be considered as a contemporary phase of what the early stages of adaptive radiation might look like (Yoder *et al.*, 2010). Experimental determination of the relative contribution of both of these factors can be achieved through rearing individuals from wild populations in a common garden and exposing those individuals to experimental treatments that mimic a key feature of habitat contrasts between ecotypes. Yet, the application of this approach to simultaneously analyze both the genetic and plastic contributions to adaptive trait differences in systems that have evolved over a contemporary time scale have rarely been employed (Ghalambor *et al.*, 2007). Although several examples of studies using this approach exist in plant and invertebrate systems, (e.g. (Weinig, 2000; Carroll *et al.*, 2001; Lee & Petersen, 2003; Bossdorf *et al.*, 2005; Colautti *et al.*, 2010), it has far less been employed in vertebrates (Robinson & Wilson, 1996; Collyer & Adams, 2007). Here we applied this approach to lab-reared offspring from parents of invasive threespine stickleback that derive from two contrasting habitats, lake and stream.

Because we investigated the phenotypic divergence in F1 offspring, maternal effects could potentially account for phenotypic divergence that is not explained by treatment. Previous work suggests, however, that maternal effects play only a minor role in explaining morphological differences between lake-stream stickleback from the Misty Lake system in Canada, where F2 fish reared in the lab were found to be phenotypically very close to those of F1 lab reared fish (Berner *et al.*, 2011). This was similarly true for other sympatric Canadian ecotypes that differ along the limnetic-benthic axis (Hatfield, 1997). Nevertheless, even the small phenotypic difference between F1 and F2 fish in both Canadian systems may contribute largely to the difference observed in the evolutionary younger studied Swiss system. Yet a recent comparative study suggests that the parapatric phenotypic differentiation in the Lake Constance system can be as divergent as the Canadian systems, depending on the traits studied (Lucek et al. 2013). Specifically gill raker length was found to be more divergent in the Lake Constance system. Although divergence in body shape was found to be lower in Constance than in the Canadian systems, the results cannot directly be compared as different sets of landmarks were used. Therefore, our experimental and statistical design is suited to quantify for each investigated trait the contribution

of plasticity and genetic predisposition to ecotypic divergence, with the caveat that maternal effects cannot be completely ruled out.

Overall we find the phenotypic differentiation among our experimental groups to be similar with that observed between the adult wild lake-stream populations, as suggested by the pairwise Mahalanobis distances and the relative classification success (Fig 1 & 3). By comparing the multivariate axes separating either source populations or the food treatments of our experimental fish, we successfully estimate the relative contribution of both phenotypic plasticity and genetic predisposition that lead to the phenotypic divergence between wild lake and stream populations (Fig 4). For both wild and experimental fish, differentiation occurs mainly in functionally relevant trophic morphology that is predicted to facilitate exploiting alternative habitats (Anker, 1974; Walker, 1997; Robinson, 2000; Wark & Peichel, 2010). This suggests that contemporary ecotype formation has occurred as a consequence of concerted action of divergent adaptation to different habitats and adaptive phenotypic plasticity (Fig 3, Ghalambor *et al.*, 2007). This is apparent when experimental and YOY individuals were projected onto the axis that separates the wild adult populations (Fig 3). All projected groups follow the predicted direction, underlying the importance of the combined effect of phenotypic plasticity and additive genetic variation that leads to overall phenotypic divergence. For morphometric shape, experimental individuals deriving from the lake even exceed the wild-type adult lake phenotype. This may indicate the limitations of our experimental setup where we focus on a single, yet major axis of parapatric divergence, i.e. on feeding ecology, whereas additional agents of selection may result in the overall observed phenotypic divergence among the wild adult populations such as divergence in predation regimes (Reimchen, 1994; Zeller et al., 2012a, Lucek et al. 2013).

Phenotypic divergence in wild and experimental populations

The observed phenotypic differentiation among wild populations corroborates evidence for ecotypic differentiation among the wild populations studied here (Roy *et al.*, 2010; Lucek et al., 2012a; 2013), and more generally within the Lake Constance region (Berner *et al.*, 2010). Ontogenetic trajectories are likely to differ given the inversed size difference among YOY and adults in the wild, which has previously been suggested (Lucek et al., 2012a; Moser *et al.*, 2012). The observed increase in body depth among wild stream fish, is thought to be associated with increased maneuverability and burst swimming, required for predator avoidance in structured habitats, whereas a smaller body depth in lake fish may facilitate sustained swimming performance, facilitating foraging in open water (Walker, 1997; Wark & Peichel, 2010; Hendry *et al.*, 2011). Other common and adaptive features of lake dwelling and plankton feeding stickleback ecotypes are elongated heads with larger eyes and longer gill rakers (Robinson, 2000; Adams & Huntingford, 2004; Wund *et al.*, 2008; Willacker *et al.*, 2010). Differentiation between lake and stream stickleback in these traits occurs already in YOY, but is overall less developed than in adults (Fig 1).

Our experiment suggests that the ecotypic differentiation in head shape and trophic morphology is rather genetically determined, whereas differentiation in body depth is mainly environmentally induced, with fish from the plankton treatment being more streamlined than fish from the benthos treatment, irrespective of source population (Fig 1d & 4a). The additive effects of both source populations and treatments result in similar phenotypic differentiation as found in the wild populations (Fig 3). This suggests that adaptation to different food sources is an important driver of phenotypic divergence in the wild. Moreover it implies that the concerted action of divergent genetic predisposition and adaptive plasticity can lead to the onset of ecological diversification (Prentis *et al.*, 2008; Yoder *et al.*, 2010; Thibert-Plante & Hendry, 2011; Westley, 2011).

In contrast to morphometric shape, environmentally induced changes are the main contributors of the observed phenotypic variation using linear morphology (Fig 2), where the significant interactions for PC scores suggest different multivariate reaction norms between the source populations (Proulx & Magnan, 2004). This is a common finding among studies using linear morphology to investigate ecotype formation in postglacial freshwater fish (Robinson & Wilson, 1996; Proulx & Magnan, 2004; Adams & Huntingford, 2004). The differences between morphometric shape and linear morphology may reflect the distinctive way in which the covariance structure was calculated for each type of phenotypic data. Furthermore, each approach may differ in its ability to isolate size effects and hence to disentangle heritable or plastic effects on size-dependent traits or on size itself.

Our results contrast in an interesting way with studies on older stickleback ecotypes in the Misty Lake system on Vancouver Island, Canada that likely evolved after the last ice age ~12'000 years ago (Thompson *et al.*, 1997). There, differentiation in body shape has been found to be genetically determined (Sharpe *et al.*, 2008; Berner *et al.*, 2011), whereas the shape of the snout was relatively plastic (Berner *et al.*, 2011). Several factors may account for these differences. First, the adaptive potential and genetic constraints of the ancestral populations may differ between the populations that we studied and those from Canada, resulting in different evolutionary responses to similar selection (Jones et al., 2012b). Indeed, the traits that underlie ecotypic lake-stream divergence in Switzerland were found to differ from the Misty system. Specifically, Canadian lake populations have a higher number of gill rakers than stream populations but not in Switzerland, where lake populations have longer gill rakers (Kaeuffer *et al.*, 2012; Ravinet et al., 2013b, Lucek et al. 2013). Second, the colonization history differs between the Misty Lake and the Lake Constance system. Whereas the former derives directly from marine ancestors (Thompson *et al.*, 1997), the latter most likely originates from populations with a history in freshwaters (Lucek *et al.*, 2010). Thus ancestral plasticity may have been different for different traits between the Misty and the Constance stickleback, leading to different trajectories of phenotypic divergence (West-Eberhard, 2003; Thibert-Plante & Hendry, 2011). Third, if ecotype formation in stickleback causes phenotypic canalization or shifts in the reaction norms, divergent genetic determination of body depth may not have yet occurred given the age of the Lake Constance

system. Plasticity can be maintained if it is not costly and selection is relatively weak or fluctuating (Lande, 2009; Thibert-Plante & Hendry, 2011).

Adaptive divergence

Differences between environments can induce divergent selection on functional and biomechanical traits, resulting in ecological divergence (Nosil *et al.*, 2009). The adaptive value of our distinct lake and stream phenotypes can be assessed through comparisons with other stickleback ecotypes and fish species with analogous adaptations (Barel, 1983; Caldecutt & Adams, 1998). The observed shape differences in both experimental and wild caught individuals have consequently functional morphological implications related to feeding: the terminal mouth in wild and lab reared lake sticklebacks together with an anterior shift of the maxilla and a smaller suspensorium are predicted to result in a reduction of suction force compared to the anatomy of wild and lab reared stream sticklebacks (Caldecutt & Adams, 1998). In addition, in cichlid fish, limnetic plankton feeding species tend to generally have elongated and slender heads (Barel, 1983). This was observed in our experimental plankton treatment, irrespective of source population, and in both age classes of the wild type lake population, where individuals showed decreased head depth and a terminal mouth relative to individuals from either the benthic treatment or the wild type stream population. Together, these differences result in phenotypes that may be more suitable for ram type feeding in lake fish compared to more suction type feeding in stream fish (Caldecutt & Adams, 1998) predicting fitness advantages for each ecotype in its own environment (Lucek et al., 2012a).

The observed phenotypically plastic difference for experimental individuals in body depth, with limnetic fed fish being more streamlined than their benthic fed counterparts, is consistent with the phenotypic differentiation between the wild adult populations, which feed either predominantly on limnetic prey in the lakes or on benthic prey in streams (Lucek et al., 2012a; Moser *et al.*, 2012). The more streamlined plankton feeding phenotype is furthermore in line with observations in other wild stickleback populations that differ along the benthic-limnetic axis (Walker, 1997; Hendry & Taylor, 2004; Willacker *et al.*, 2010) and may result in an increased sustained swimming capability (Walker, 1997; Blake *et al.*, 2005). Indeed, the observed plastic response in body depth between our experimental food treatments could be caused by different foraging behavior as swimming effort differed between treatments, i.e., feeding on live zooplankton required an increased sustained swimming capability compared to feeding on benthic insect larvae. Taken together, the observed phenotypic differences are consistent with additive effects of adaptive plasticity and divergent adaptation. Because former genetic analyses showed that the lake and stream populations investigated here are very closely related, and in fact are more closely related to each other than to other Swiss populations (Lucek *et al.*, 2010; Berner *et al.*, 2010), the lake-stream divergence observed here likely evolved *in situ*. Hence, we have shown ecotypic differentiation that has evolved within less than 140 years among populations of an invasive species that occupy distinct habitats.

The observed and potentially heritable phenotypic differentiation related to habitat and feeding ecology mark the transition from invasion and niche expansion with establishment in a new environment towards populations that undergo divergent adaptation (Hendry *et al.*, 2000; Prentis *et al.*, 2008). Such adaptive evolutionary divergence might be a precursor of ecological speciation (Nosil, 2012). Phenotypic plasticity may, on the other hand, delay or even prevent further divergence by shielding the genome from divergent selection depending on the underlying selective regime (Price *et al.*, 2003; Ghalambor *et al.*, 2007; Thibert-Plante & Hendry, 2011). The formation of divergent ecotypes can have further implications for the impact of the invasive species on native species and the ecosystem itself: By adapting to effectively exploiting different niches, different stickleback ecotypes are likely to introduce different selection pressures on their prey, competitors and predators and may hence induce divergent evolutionary responses in other organisms (Vellend *et al.*, 2007; Shine, 2012). Indeed, experimental evidence suggests that divergent stickleback ecotypes from within the native range, are able to affect the community composition of lower trophic levels in distinct ways (Harmon *et al.*, 2009).

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Supplement

Table S1: *Description of the landmarks used for shape analysis.*

Landmark	**Description**
1	Anterior extent of maxilla
2	Posterior extent of maxilla
3	Insertion between quadrate and articular bone
4	Insertion between 2nd and 3rd suborbital
5	Anterioventral extent of sphenotic at orbit
6	Center of the eye
7	Posterior extent of supraoccipital
8	Posteriodorsal extent of operculum
9	Anterioventral extent of operculum
10	Caudoventral extent of interoperculum
11	Anterior insertion of the first dorsal spine
12	Anterior insertion of the second dorsal spine
13	Anterior extent of the dorsal fin rays
14	Posterior extent of the dorsal fin rays
15	Posterioventral extent of the caudal fin
16	Posterior extent of the anal fin rays
17	Anterior extent of the anal fin rays
18	Dorsal insertion of the pelvic spine
19	Dorsal extent of the pelvic fin

Table S2: *Loadings based on linear discriminant (LD) analyses for each linear measurement for wild caught adults, young of the year (YOY) and experimental individuals.*

	Wild		Experiment	
Trait	Adults	YOY	Treatment	Source
Body depth	0.763	-0.298	-3.466	-0.502
Head length	0.793	0.787	-0.250	3.815
Head depth	-1.557	0.087	2.376	-1.064
Eye diameter	-1.622	-2.339	-3.882	-2.124
Upper jaw length	-0.874	-1.914	2.398	1.082
Lower jaw length	3.765	2.045	-3.832	1.022
Snout length	-2.611	-2.234	0.784	-5.407
Gape width	0.857	5.869	2.187	-5.012
Length of 2nd gill raker	-1.613	2.928	1.408	1.982
Gill arch length	-0.977	-0.606	2.112	-1.037

Table S3: *Significances for linear measurements for both wild caught adults and young of the year (YOY) based on pairwise t tests. Both t and p values are given as well as the mean (ø) values for both populations. Significant p values are highlighted in bold.*

				Adults			**YOY**	
Trait	$t_{1,139}$	***p***	**ø lake**	**ø stream**	$t_{1,82}$	***p***	**ø lake**	**ø stream**
Standard length	9.730	**<0.001**	56.18	48.39	-12.063	**<0.001**	22.39	34.37
Body depth*	-3.984	**<0.001**	-0.001	0.420	1.482	0.142	0.227	0.133
Head length*	0.572	0.569	-0.074	-0.154	-5.579	**<0.001**	-0.313	0.059
Head depth*	3.025	**0.003**	0.091	-0.189	0.506	0.615	0.043	0.018
Eye diameter*	0.730	0.468	-0.076	-0.106	-8.153	**<0.001**	-0.520	-0.275
Upper jaw length*	-0.242	0.809	0.027	0.043	1.807	0.075	0.023	-0.036
Lower jaw length*	-1.991	**0.049**	-0.072	0.065	-4.537	**<0.001**	-0.175	-0.003
Snout length*	2.695	**0.008**	0.043	-0.139	1.318	0.192	0.101	0.052
Gape width*	-0.049	0.961	-0.028	-0.025	-8.720	**<0.001**	-0.010	0.236
Length of 2nd gill raker*	3.975	**<0.001**	0.012	-0.093	-3.287	**<0.001**	-0.039	0.022
Gill arch length*	3.398	**0.001**	0.016	-0.160	-2.238	**<0.001**	-0.077	0.001
# Gill raker	-0.697	0.487	18.25	18.41	-1.915	0.059	17.95	18.41

* size corrected traits

Table S4: *p values for all linear size-corrected morphological traits separately and combined using principal components (PC; axes 1-3 indicated). In addition the p values for the three leading PC axes of geometric shape are given. Results for the gill raker number are shown separately. The significance levels are based on mixed linear models with source population and treatment as response variable and family as random effect using a backward elimination procedure and a type II F tests. Significant values are highlighted in bold, values 0.1 > p > 0.05 are highlighted in italic. The main contributing response effect is indicated. See main text for abbreviations and figure S2 for details.*

Trait	**Treatment df = 1,426**	**Source Population df = 1,11**	**Interaction df = 1,426**	**Effect**
Single traits				
Body depth	**<0.001**	0.767	0.133	Plastic
Head length	**<0.001**	0.932	0.123	Plastic
Head depth	0.135	0.471	*0.053*	None
Eye diameter	**<0.001**	0.682	0.532	Plastic
Upper jaw length	0.535	0.400	0.206	None
Lower jaw length	**<0.001**	0.578	0.102	Plastic
Snout length	**0.025**	0.152	*0.088*	Plastic
Gape width	**0.004**	0.206	**<0.001**	Reaction norms differ
Length of 2nd gill raker	0.899	0.445	0.658	None
Gill arch length	*0.077*	0.734	0.398	None
# Gill raker	*0.078*	0.510	0.889	None
Multivariate trait combination				
PC1 (48.9%) – linear measures	**<0.001**	0.881	0.999	Plastic
PC2 (16.0%) – linear measures	**0.013**	0.343	**0.014**	Reaction norms differ
PC3 (10.3%) – linear measures	**<0.001**	0.686	**0.014**	Reaction norms differ
PC1 (29.3%) – shape	0.872	0.119	0.721	None
PC2 (17.1%) – shape	*0.077*	0.307	0.814	None
PC3 (10.6%) – shape	**0.047**	0.629	0.577	Plastic

Table S5: *Standardized relative loadings for each landmark coordinate based on canonical variate (CV) analyses. Loadings ≥0.5 are highlighted in grey.*

	Wild		Experiment	
	Adults	**YOY**	**Treatment**	**Source**
x1	0.001	0.361	0.232	0.630
y1	0.005	0.123	0.208	0.003
x2	1.000	0.268	0.171	0.649
y2	0.539	0.143	0.549	0.448
x3	0.536	0.685	0.283	0.208
y3	0.441	0.123	0.270	0.078
x4	0.028	0.083	0.101	0.130
y4	0.288	0.476	1.000	0.664
x5	0.278	0.211	0.771	0.825
y5	0.681	1.000	0.534	0.881
x6	0.348	0.340	0.381	0.356
y6	0.755	0.238	0.281	0.146
x7	0.132	0.020	0.159	0.076
y7	0.300	0.190	0.873	0.113
x8	0.451	0.434	0.050	1.000
y8	0.178	0.111	0.091	0.011
x9	0.209	0.222	0.452	0.274
y9	0.259	0.247	0.768	0.294
x10	0.150	0.231	0.024	0.517
y10	0.458	0.822	0.135	0.771
x11	0.190	0.080	0.044	0.065
y11	0.076	0.266	0.477	0.034
x12	0.099	0.056	0.028	0.232
y12	0.232	0.199	0.069	0.264
x13	0.199	0.050	0.068	0.069
y13	0.199	0.676	0.559	0.266
x14	0.002	0.087	0.020	0.073
y14	0.082	0.163	0.015	0.200
x15	0.197	0.103	0.026	0.200
y15	0.124	0.096	0.014	0.094
x16	0.008	0.117	0.021	0.069
y16	0.330	0.257	0.124	0.383
x17	0.101	0.028	0.215	0.170
y17	0.174	0.486	0.483	0.371
x18	0.193	0.020	0.275	0.148
y18	0.030	0.088	0.299	0.214
x19	0.313	0.168	0.206	0.018
y19	0.016	0.258	0.132	0.041

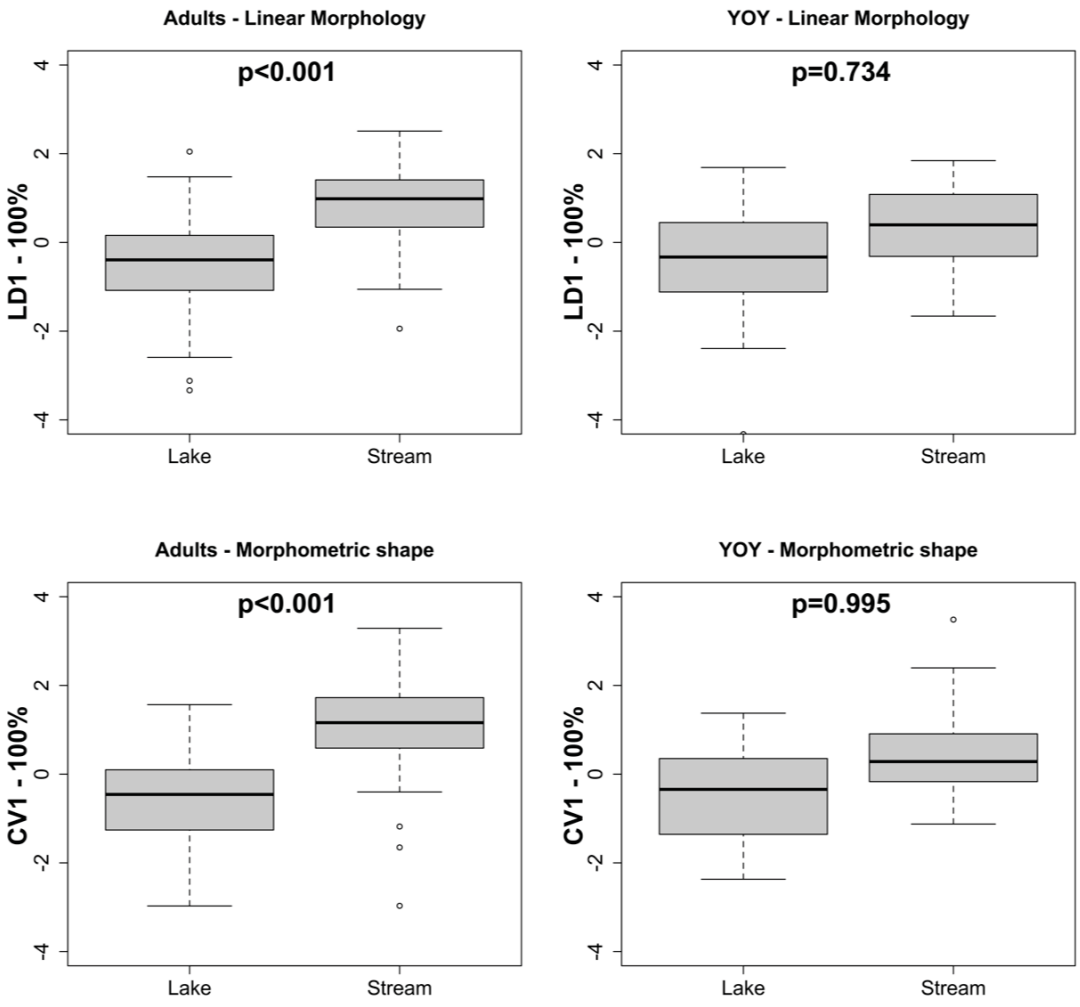

Figure S1: *Phenotypic differentiation between wild caught adults (left) and young of the year (YOY; right) for linear morphology based on linear discriminant scores (LD; top) and morphometric shape differentiation based on canonical variate scores (CV; bottom). Statistical significances were either estimated using a MANOVA for linear morphology or using a bootstrap approach for morphometric shape (see main text for details).*

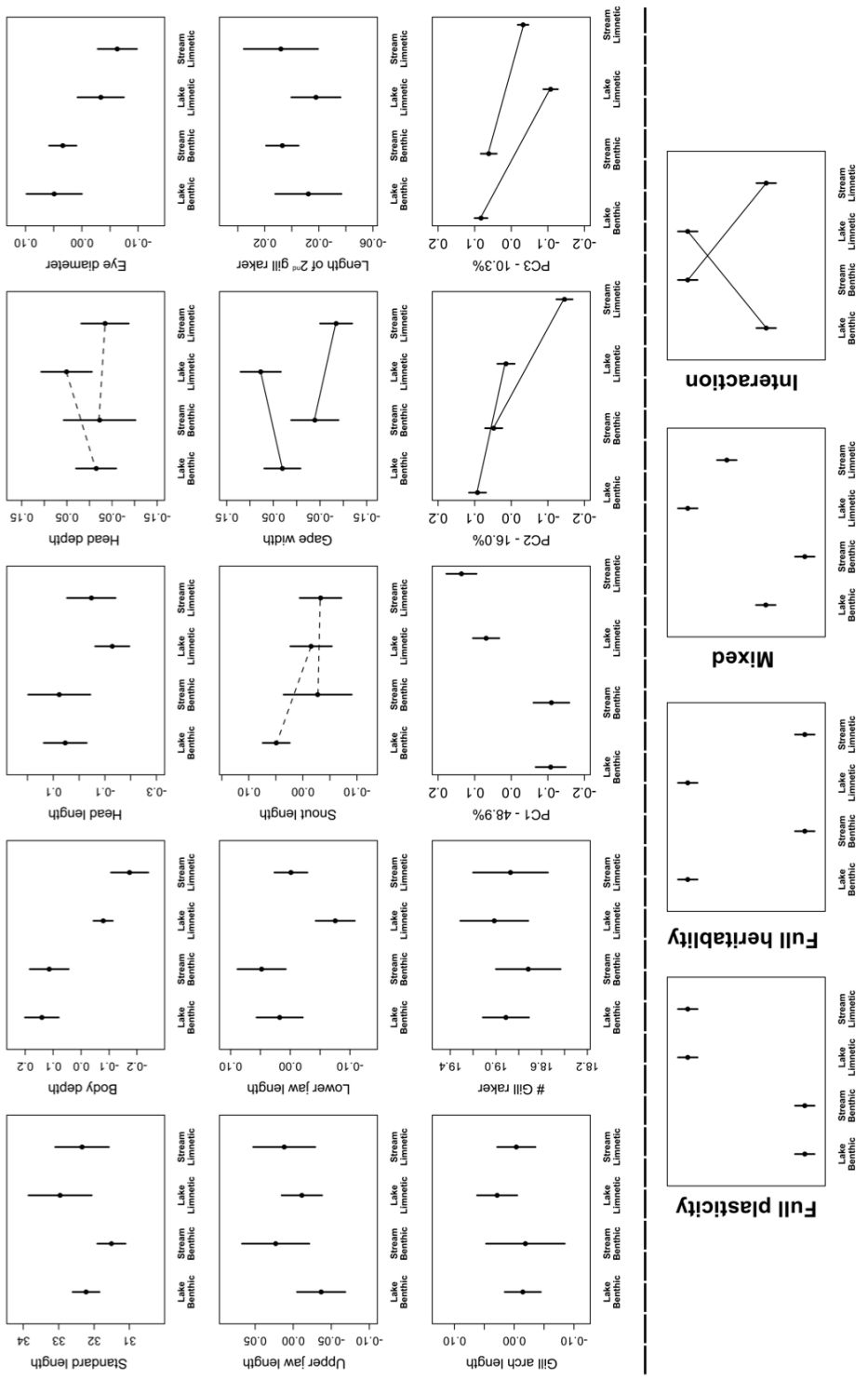

Figure S2: Family means (± 1 SE) for all linear traits measured including gill raker number and the three leading PC axes separated by treatments (benthos and plankton) and source populations (lake and stream). The bottom panel shows four hypothetical cases assuming from left to right: full plasticity, full heritability, an intermediate with no significant interaction and an interaction of both plasticity and heritability. Connections between means represent cases with a significant interaction ($p < 0.05$; solid lines) or with a statistical tendency ($0.1 < p < 0.05$; dashed lines), see Table S3 for details.

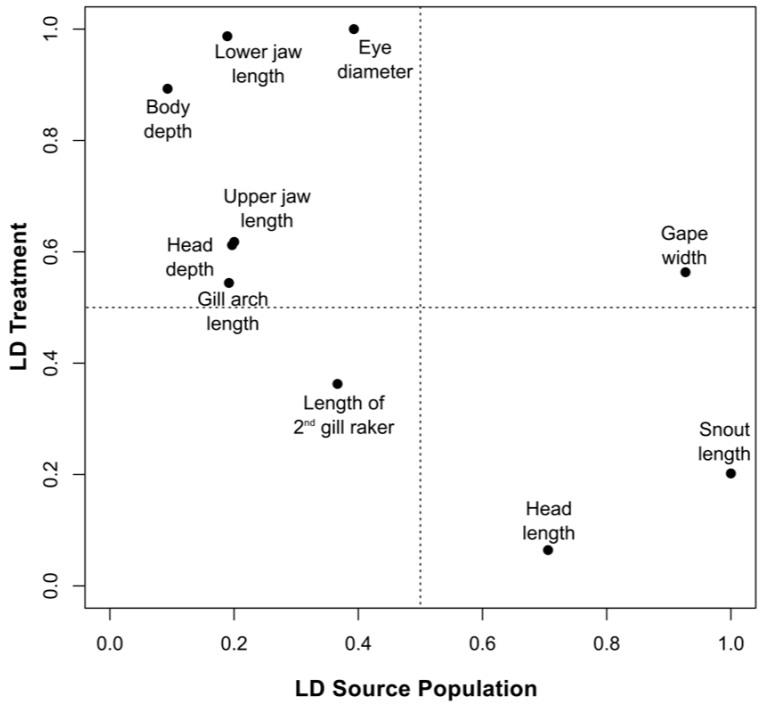

Figure S3: *Relative trait loadings of the leading linear discriminant (LD) axis based on linear morphological data from experimental individuals using either source population (lake, stream) or treatment (plankton, benthos) as grouping variable. Dashed lines indicate an arbitrary cutoff value of 0.5.*

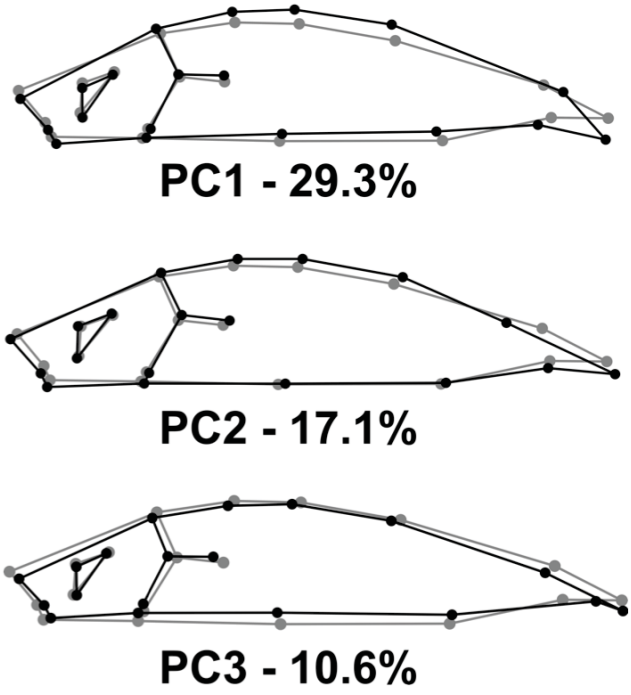

Figure S4: Morphometric shape differences along the three leading PC axes for experimental individuals. Deformations (black) show the differences from the consensus (grey).

Chapter 6

Quick divergence but slow convergence during parallel ecotype evolution: time, historical contingency and parallelism in lake and stream stickleback pairs of variable age

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Abstract

When genetic constraints restrict phenotypic evolution, diversification can be predicted to evolve along so-called lines of least resistances. To address the importance of such constraints and their resolutions, studies of cases of parallel phenotypic divergence that differ in their age are valuable. Here we investigate the parapatric evolution of six independent lake and stream stickleback systems from Iceland and Switzerland, ranging in age from a few decades to several millennia. Using phenotypic data, we test for parallelisms in ecotypic divergence between lake and stream in parapatry and compare patterns to an ancestral-like marine population. We find strong and consistent phenotypic divergence, for both the parapatric lake-stream systems and for the marine-freshwater transition. Interestingly, ecotypic divergence in low dimensional phenotype space (i.e. single traits) is rapid and seems to be often completed within 100 generations. This may partly reflect phenotypic plasticity during the colonization of novel habitats. Yet, the dimensionality of ecotypic divergence was highest in our oldest systems and only there parallel evolution of unrelated ecotypes was strong enough to overwrite phylogenetic contingency. Interestingly also, the dimensionality of divergence in different systems varies between trait complexes, suggesting different constraints and evolutionary pathways to their resolution among freshwater systems.

Introduction

If natural selection is the principal force governing evolutionary change, divergence among populations can be seen as the tracking of alternative adaptive peaks on the underlying fitness landscape (Wright, 1932; Lande & Arnold, 1983; Steppan *et al.*, 2002; Arnold *et al.*, 2008). The degree of divergence is then expected to depend on the time that was available for selection to act, on the strength of selection, the topology of the fitness landscape and the amount of adaptive standing genetic variation within each population. All of these factors may affect both the rate and the direction of evolution. Additionally, the strength of selection and/or the fitness landscape itself may fluctuate through time due to environmental variation (Jones *et al.*, 2004; Arnold *et al.*, 2008). Both genetic drift and selection can reduce standing genetic variation, which may finally lead to different evolutionary outcomes across replicated cases of population divergence, even when selection is acting in a parallel manner across replicates(Barrett & Schluter, 2008). Consequently, strong parallel or convergent evolution is only expected if the selective regime, the relative level of standing genetic variation as well as the segregating alleles themselves are similar (Langerhans & DeWitt, 2004; Kaeuffer *et al.*, 2012).

Evolution towards adaptive peaks can be seen as the progression along "lines of least resistances" or g_{max}, which can be quantified as the leading eigenvector of the genetic variance-covariance matrix G (Schluter, 1996). Biologically, this axis comprises most of the genetic variation, shaped by selection and drift, and reflects the underlying genetic constraints within a population (Marroig & Cheverud, 2005). Different G matrices can be compared by calculating the angle θ between different g_{max} (Schluter, 1996), where especially short-term evolution is predicted to follow the direction of this leading axis. Through time, selection may change the direction of g_{max} towards an existing or a new optimum on the adaptive landscape, e.g. during the colonization of new environments (Bacigalupe, 2009; Eroukhmanoff & Svensson, 2011). Genetic drift, bottlenecks and mutation may all change the G matrix and hence g_{max} over time (Chapuis *et al.*, 2008).

In the absence of quantitative genetic data, the G matrix can be approximated by the P matrix, which is based on phenotypic data from wild populations (Arnold *et al.*, 2008). Here, P is defined as the combination of the genetic and environmental covariance matrices, i.e. G + E (Lande, 1979; Arnold & Phillips, 1999), where both may furthermore interact (G x E; Falconer, 1989). Consequently, P matrices potentially include phenotypically plastic traits, that are differentially expressed in distinct environments (Pigliucci *et al.*, 1999). The leading eigenvector of a P matrix (p_{max}) may therefore serve as an overall measure of phenotypic variation observed in the wild, accounting for both genetic and environmental effects. However, evolutionary patterns of p_{max} differ between plastic and genetically determined traits, which becomes apparent when studying parallel cases of phenotypic divergence along a temporal gradient. On the one hand, adaptive phenotypic plasticity can promote rapid phenotypic differentiation between distinct environments (Ghalambor *et al.*, 2007; Thibert-Plante & Hendry, 2011; Svanbäck & Schluter, 2012). Depending on the selective regime, genetic assimilation may reduce plasticity over time, while the

phenotypes that were initially produced by plasticity are retained (Lande, 2009). Consequently p_{max} of different replicated systems that vary in their age should align, i.e. show a small or zero angle θ between them, if each system has similar amounts and kinds of standing genetic variation and experiences a comparable selective regime. However, because phenotypic plasticity itself can evolve as an adaptation to a novel environment, p_{max} may show a gradual evolution from an ancestral state over a few generations before integration occurs (Lande, 2009; Svanbäck & Schluter, 2012; Draghi & Whitlock, 2012). On the other hand, assuming a comparable adaptive landscape and selective regime among replicates, θ between mainly genetically determined p_{max} should evolve over time through selection and drift (Schluter, 1996). Here, θ is expected to subsequently increase over time between an ancestral p_{max} and the p_{max} of a derived population that is evolving towards a new adaptive optimum (Schluter, 1996; 2000). This has been shown in a comparative study among several vertebrate taxa, based on genetic distances to establish divergence time (Schluter, 1996). However, founder effects can induce rapid evolutionary changes during the colonization of new environments, changing the evolution of the underlying G matrix (Bacigalupe, 2009). Moreover, depending on the level of gene flow, the genetic differentiation from an ancestral population may remain low, despite a significantly diverged G matrix (Calsbeek *et al.*, 2011; Eroukhmanoff & Svensson, 2011).

In threespine stickleback (*Gasterosteus aculeatus* species complex) the ancestral marine population repeatedly colonized freshwater throughout its distribution shortly after the last glacial maximum and subsequently adapted to different habitats such as streams and lakes, forming phenotypic and ecologically divergent populations and in some cases even sympatric or parapatric species (Bell & Foster, 1994; McKinnon & Rundle, 2002). Especially the parallel evolution of distinct parapatric lake-stream pairs has made this species complex an excellent system to investigate replicated ecological speciation. However, most studies use evolutionary relatively old systems and are limited to restricted geographical areas (Hendry & Taylor, 2004; Berner *et al.*, 2008; Kaeuffer *et al.*, 2012; Ravinet *et al.,* 2013b, but see Berner *et al.*, 2010). In contrast, some lake-stream systems became only recently available to stickleback due to contemporary translocations (Lucek *et al.*, 2010; 2012a) or the creation of artificial lakes (Kristjánsson *et al.,* 2002a; Kristjánsson, 2005). Hence, stickleback provide a rare opportunity to study the evolution of parapatric divergence along the lake-stream habitat axis over a wide timescale, ranging from decades to millennia.

Here, we study an exceptional system of replicated parapatric lake-stream stickleback from Switzerland and Iceland that are between 50 and 10,000 years old in relation to their putative ancestral marine population. Using the temporal gradient, we test if phenotypic divergence emerges rapidly after the colonization of novel environments and whether the underlying evolutionary trajectories evolve themselves over time as suggested by Schluter (1996). If phenotypic differentiation is mainly genetically based, we predict a gradual increase in the overall angle θ between the ancestral marine and the derived freshwater p_{max} over time. Thus, parapatric divergence between distinct freshwater habitats should similarly increase with time. Alternatively, if the underlying traits are

mainly plastic, or if evolution was rapid, p_{max} should align independently of evolutionary age. Additionally, the geographic scale coupled with the very different colonization histories of Iceland and Switzerland (Ólafsdóttir et al., 2007c; Lucek *et al.*, 2010) allows us to test for true parallel evolution, i.e. if phenotypic evolution in similar environments proceeds along a common evolutionary trajectory and to which degree these evolutionary trajectories and the extent of phenotypic divergence may differ due to different historical contingencies.

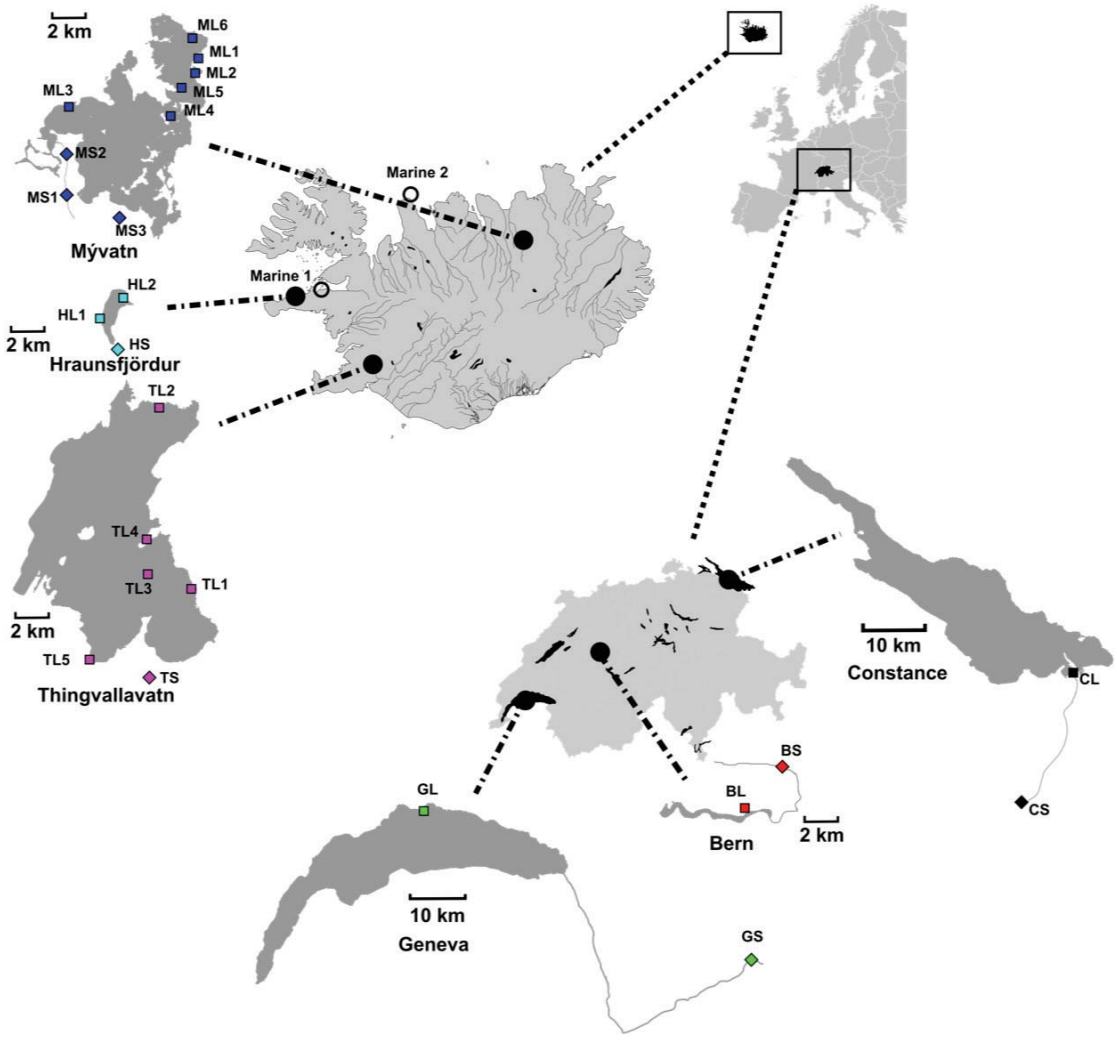

Figure 1: *Sampled lakes and corresponding sampling sites (squares: lake populations; diamonds: stream populations; circles: marine populations) for both Iceland (top) and Switzerland (bottom).*

Material and Methods

Sample collection

We studied three Swiss lake-stream systems in the invasive range of stickleback that differ in their ages of stickleback colonization (Bern (Lake Wohlen): ~50 yrs, Constance: 140 yrs, Geneva: 140 yrs) and represent either independent introductions from different freshwater lineages (Constance and Geneva) or a case of recent admixture of these lineages (Bern; see Lucek *et al.*, 2010 for details). In addition, we studied three Icelandic lake-stream systems that differ in their geological age (Mývatn: 2500 yrs, Thingvallavatn: 8000-10'000 yrs; Saemundsson, 1992; Einarsson *et al.*, 2004) or are man-made (Hraunsfjördur: 50 yrs; Kristjánsson et al., 2002b) and have been colonized by stickleback from ancestral marine populations. We sampled two Icelandic marine populations (Table S1), representing the presumed ancestral state of freshwater stickleback (Mäkinen *et al.*, 2006). Because Icelandic lake stickleback have formed phenotypically divergent substrate-associated ecotypes (Kristjánsson et al., 2002b), we sampled several locations in each lake, and all potential habitats (see Figure 1 and Table S1 for sampling locations). Icelandic samples were obtained between August and September 2010 using minnow traps and by hand netting. Individuals from Switzerland were collected using the same methods in 2007 and 2008 (Lucek *et al.*, 2010). In all cases, stream stickleback populations were obtained from inflowing streams (Table S1). All fish were sacrificed with an overdose of clove oil and stored in 70% ethanol. In addition, a fin clip was taken for genetic analysis and preserved in absolute ethanol. Sample size per site ranged from 17 to 62 (mean: 35 ± 10 SD) with a total of 918 individuals from 26 sites (Figure 1, Table S1). Altitudinal difference and pairwise waterway distance between each stream site and the inflow of the stream into the lake were measured using GOOGLEEARTH (Google, USA).

Genetic analysis

We extracted DNA for the Marine 1 population and all freshwater individuals from all sites, except for Mývatn, where only one of the three stream sites (MS1) was available for genetic analysis (N_{Total} = 794, Table S1). DNA was extracted using a 10% Chelex solution, following the manufacturers protocol (Biorad, California, USA). In a few cases, additional individuals were included for which no phenotypic data was collected (Table S1). We amplified ten microsatellite markers in one multiplex kit following the protocols of Raeymaekers *et al.* (2007). Detailed information on the multiplexing setup and the PCR protocol are provided as supplementary methods. We visualized alleles on an ABI 3130XL and scored them with GENEMAPPER 4.0 (Applied Biosystems, Switzerland). We generated a genetic tree-like relationship among sampling sites based on their Cavalli-Sforza distances of allelic frequencies using a neighbour-joining algorithm implemented in the program PHYLIP 3.69 (Felsenstein 2012). Significance was further estimated using 1000 bootstrapped resampling replicates. Using GENODIVE 2.0 (Meirmans & Van Tienderen, 2004), we calculated pairwise F_{ST} between parapatric lake and stream populations for all systems, pooling all sampling sites within a lake. We estimated significances using 1000 replicates of the bootstrapping procedure implemented in GENODIVE. Finally, we

tested for a correlation of the obtained pairwise F_{ST} values with either the parapatric altitudinal difference or geographical distance between a stream site and the lake. Models were compared using the Akaike information criterion corrected for finite sample sizes (AICc).

Morphological data collection & analysis

We measured sixteen linear morphological traits (see Figure S1 for details), many of which are known to be associated with ecological diversification in stickleback (see Kristjánsson et al., 2002a; Mori & Takamura, 2004; Berner *et al.*, 2008 and references therein) on the left side of each fish to the nearest 0.01 mm using a digital caliper. These traits are either related to anti-predator defense (FSL - length of the first dorsal spine; DSL - length of the second dorsal spine; PSL - length of the pelvic spine; PGL - length of the pelvic girdle), feeding ecology (HL - head length; UJL - upper jaw length; SnL - snout length; SnW - snout width; ED - eye diameter) or linked to general body shape and swimming performance (SL - standard length; PGW - width of the pelvic girdle; BD1 -body depth measured after the first dorsal spine; BD2 - body depth measured after the second dorsal spine; CPL - caudal peduncle length; BLA - basal length of the anal fin; BLD - basal length of the dorsal fin; TLP - total length of the pelvic fin). In addition, we measured the length of the lower gill arch (AL) and the length of the second gill raker (GRL2), as counted from the joint of the dorsal arch bone on the first lower gill arch. Both measurements on the gill arch were done using a micrometer mounted on a dissection microscope. Both gill raker traits are related to feeding ecology (Berner *et al.*, 2008). Because all traits were significantly correlated with SL (results not shown), we size-corrected the data by using the residuals of a regression of each trait against standard length SL. Depending on the question we ask, this was either performed pooling all individuals for the overall comparison of populations or separately (i.e. for each parapatric lake-stream system and for the marine sample) for parapatric comparisons. By pooling all systems, allometric information among them can be retained if the allometric trajectories differ between study systems. This means that we retain lineage dependent allometric information via this approach.

To compare the overall morphological (hereafter phenotypic) divergence among all sampled sites and lakes, we calculated the pairwise Mahalanobis distances among sampling sites using the overall size-corrected linear phenotypic measurements. To visualize the obtained relationships among sites, we constructed a distance tree based on the obtained pairwise Mahalanobis distances.

We estimated the parapatric phenotypic divergence as pairwise parapatric P_{ST}, an analog to Q_{ST} (Spitze, 1993) based on phenotypic measurements from wild individuals, using the approach of Kaeuffer et al. (2012) using the residuals of the first principal component (PC) axis, i.e. p_{max}, of phenotypic traits that were separately size corrected for each system. Pairwise parapatric P_{ST} were furthermore calculated separately for each trait. As pointed out by several authors (Hendry, 2002; Edelaar & Björklund, 2011), P_{ST} should only be used to infer divergent selection on a trait and hence comparing it to the genetic divergence (F_{ST}) in neutral genetic marker in evolutionary young

populations that evolved from the same common ancestor. Consequently we compare P_{ST} values with their respective F_{ST} to infer divergent selection only between parapatric populations.

Because local adaptation may lead to phenotypic differentiation among populations of the same ecotype (i.e. within one habitat type; (Ravinet *et al.*, 2013b), sampling sites from lake and stream populations were pooled for all parapatric comparisons, to estimate the overall axis of phenotypic variation between habitats. Four sets of analyses were performed: one using all phenotypic traits, and three subsets containing only traits related to defense (FSL, DSL, PSL, PGL), feeding (HL, UJL, SnL, SnW, ED, GRL2, AL) or body shape and swimming performance (PGW, BD1, BD2, CPL, BLA, BLD, TLP). We calculated pairwise P_{ST} following Kaeuffer *et al.* (2012), estimating their 95% confidence intervals with a resampling approach of 1000 replicates.

To further estimate the relative contributions of *country* (Iceland or Switzerland), *system* (Bern, Constance, Geneva, Hraunsfjördur, Mývatn, Thingvallavatn), *habitat* (lake or stream) and the interaction *of system* x *habitat* on parallel diversification within freshwater and historical contingency of each trait, we estimated the percentage of non-error variance explained by each statistical model, using their respective sums of squares, based on a sequential ANOVA model (Langerhans & DeWitt, 2004; Eroukhmanoff *et al.*, 2009). Here, *country* should reflect variation explained due to different historical contingency between Switzerland and Iceland. Similarly, *system* accounts for the variation between allopatric lake-stream systems (drainage systems) and may reflect colonization history or environmental. The *habitat* term reflects the component of parapatric divergent adaptation that is replicated among systems. Finally, the *system* x *habitat* interaction should account for the combined effect and interaction of system related differences (colonization history and environmental differences) and ecotypic divergence, which we call unique diversification (Langerhans & DeWitt, 2004; Eroukhmanoff *et al.*, 2009).

We estimated the major phenotypic evolutionary trajectory (p_{max}) for each habitat and freshwater system separately as the eigenvector of the leading PC axis. To do so, we pooled individuals from all sampling sites for each habitat. Similarly, we calculated p_{max} for the marine population as the eigenvector of a PC where we pooled both sampled populations to obtain a better estimate of the putative ancestral state of stickleback. In addition, we calculated the overall p_{max} for each freshwater system, based on the eigenvector of a PC analysis, where we pooled lake and stream sites. We then compared p_{max} of two P matrices by calculating the angle θ between them follows (Schluter, 1996), where θ is the inversed cosine of the dot product of two leading eigenvectors that is divided by the summed length of both eigenvectors. We estimated θ between parapatric stickleback populations from adjacent and connected lake and stream habitats in each system. For each freshwater system we also estimated θ between p_{max} of the ancestral-like marine population and p_{max} of each habitat. In addition, we estimated θ between p_{max} for the overall parapatric system, i.e. combining all populations and habitats within each river system. The significance of θ between p_{max} of all comparisons was estimated using a bootstrap procedure with 1000 replicates following Berner (2009). The obtained values for θ were then

correlated with the age of each system using linear models. Finally, the angles θ between the overall p_{max} for each freshwater system was compared using a similar bootstrap approach with 1000 replicates. All statistical analyses were performed in R 2.15.1 (R Core Team, 2012).

Results

Genetic divergence

DNA extractions from 73 individuals, distributed among all systems, failed to amplify after three attempts and were therefore discarded from the genetic analysis. The genetic tree supports a significant differentiation among all our studied freshwater systems, which form all distinct clades, suggesting their independent origin (Figure 2a). The Marine 1 population falls furthermore in the Hraunsfjördur clade, which is consistent with the very recent origin of this system (Kristjánsson et al., 2002b; Ólafsdóttir et al., 2007b). Further genetic substructure was indicated among the different sampling sites in all Icelandic systems.

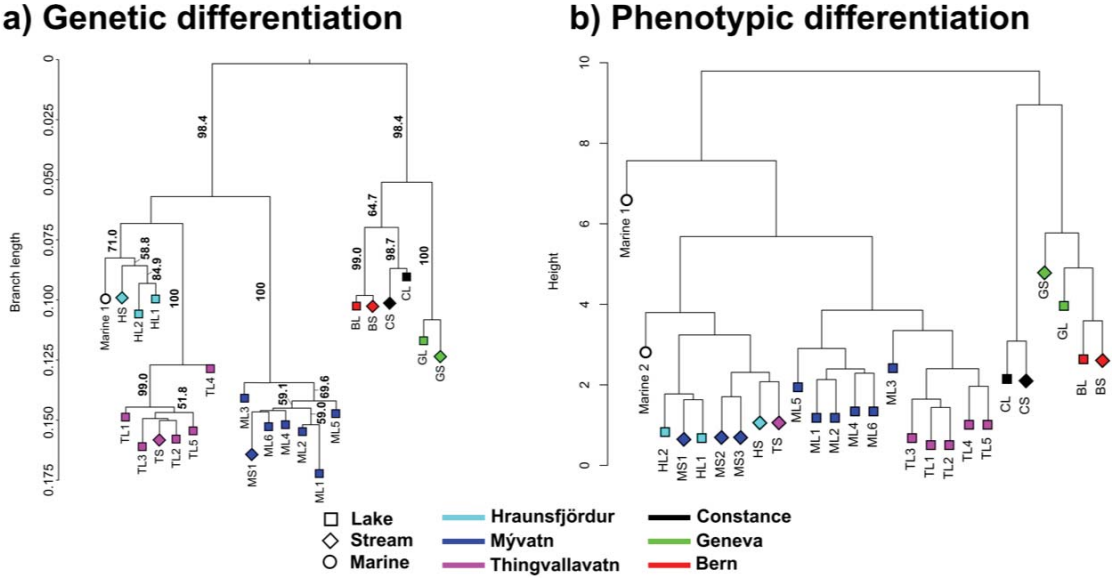

Figure 2: *Genetic and phenotypic relationship among sampling sites. Shape of tip labels indicates habitat (square: lake; diamond: stream; circle: marine) and colors represent different lake-stream systems. a) Genetic differentiation among populations based on a neighbour-joining tree using Cavalli-Sforza distances amongst sampling sites included in this study (see Figure 1), calculated from allele frequencies at 10 microsatellite loci. The tree is midpoint rooted. Numbers beside nodes indicate percent bootstrap support based on 1000 resampling replicates. Bootstrap values below 50% are not shown. b) Dendrogram of phenotypic Mahalanobis distances among all sampling sites.*

Habitat dependent parapatric genetic differentiation was highest in the Lake Geneva system in Switzerland (F_{ST} = 0.053, p < 0.001), which also showed the most distinct parapatric differences in altitude ($\Delta_{Altitude}$: 108 m) and the distance to the lake (61 km). Parapatric ecotypes in all other systems showed lower albeit significant genetic differentiation (Constance: F_{ST} = 0.018, p = 0.017; Hraunsfjördur: F_{ST} = 0.009, p = 0.006; Mývatn: F_{ST} = 0.028, p < 0.001;

Thingvallavatn: F_{ST} = 0.018, p = 0.009) except in Bern (F_{ST} = 0.000, n.s.). Pairwise F_{ST} between parapatric lake and stream populations were significantly correlated with both altitudinal differences between sites (R^2 = 0.823, $F_{1,4}$ = 18.6, p = 0.013) and distance to the lake (R^2 = 0.784, $F_{1,4}$ = 14.5, p = 0.019). These two factors were also significantly correlated with each other (R^2 = 0.922, $F_{1,4}$ = 47.2, p = 0.002) and fitted the linear model equally well (ΔAICc = 1.22).

Historical contingency and parapatric divergence in freshwater

The trait based ANOVA models all explained a significant amount of phenotypic variation (all p < 0.001, results not shown; Table 1), where the amount of variation explained by historical contingency at both geographical levels, countries and river system was generally significant and relatively high (*country*: 37.4% ± 23.0%; *system*: 35.1% ± 18.4; Table 1). Differences between stickleback in Iceland and Switzerland where most strongly different in defense related and to a lesser extent in feeding related traits while variation explained by river *system* was highest in body shape related traits. Habitat type alone explained only a small fraction of the variance (4.4% ± 5.4%). Traits TLP and BLA had the largest amount of variance explained by habitat The *system* x *habitat* interaction explained a higher significant fraction of variation (23.1% ± 19.0%), suggesting large system specific components of parapatric lake-stream divergence especially for feeding, but to a lesser extent also for body shape related traits.

The occurrence and extent of individual trait based parapatric phenotypic divergence (P_{ST}) differed among systems and countries (Figure 3a). P_{ST} commonly exceeded F_{ST} in the two oldest lakes in Iceland. These lakes, Mývatn and Thingvallavatn, showed the highest numbers of significantly differentiated traits with P_{ST} exceeding F_{ST} in 14 out of 18 cases for both lakes. However, even in the 50 years young Hraunsfjödur this was still true for 6 traits. In Switzerland, significant trait specific P_{ST}s were only observed in the slightly older Constance and Geneva systems, especially for defense traits (6 and 3 traits respectively), whereas P_{ST} did not significantly exceed F_{ST} in any of the traits in the 50 years young lake Wohlen in the Bern system. The 95% confidence intervals tended to be higher among the younger Swiss systems, suggesting larger phenotypic variation. P_{ST} based on the PC1 scores calculated in a PCA with all traits combined, exceeded the genetic differentiation significantly, i.e. the 95% confidence interval for P_{ST} did not overlap with the F_{ST} value, in the two old Icelandic lakes, Mývatn and Thingvallavatn and one of the two older Swiss systems, Lake Geneva (Figure 3b). Once the functional trait groups were analyzed in separate PCA, P_{ST} for defense related morphology exceeded F_{ST} in four cases. Only the two youngest systems, Bern and Hraunsfjördur were not differentiated. Divergence in feeding related morphology exceeded F_{ST} only in one of the two oldest lakes, Mývatn. For traits related to body shape and swimming performance finally, P_{ST} exceeded F_{ST} values in all Icelandic systems but in none of the systems in Switzerland. The magnitude of P_{ST} among parapatric ecotypes was not statistically associated with the altitudinal difference, with the waterway distance between sites or the age of a system for any trait combinations (all p > 0.1, results not shown).

Table 1: *Non-error variance components based on an ANOVA model with Country (Iceland, Switzerland), System (Bern, Geneva, Constance, Hraunsfjördur, Mývatn, Thingvallavatn), Habitat (Lake, Stream) and the interaction System x Habitat with their respective F value, degrees of freedom and significances (p) for each trait (see text for details). The R^2 values furthermore indicate the overall statistical support for each model (all $p < 0.001$).*

	Trait	R^2	Country [%]	$F_{1,799}$	p	System [%]	$F_{4,799}$	p	Habitat [%]	$F_{1,799}$	p	System*Habitat [%]	$F_{5,799}$	p
Feeding	HL	0.174	32.7	55.18	<0.001	47.2	19.90	<0.001	0.0	0.00	0.949	20.1	6.78	<0.001
	ED	0.403	60.9	329.08	<0.001	15.0	20.27	<0.001	9.2	49.67	<0.001	14.9	16.05	<0.001
	SnL	0.201	5.2	4.34	0.038	30.3	6.31	<0.001	4.7	3.90	0.049	59.8	9.95	<0.001
	UJL	0.160	33.9	60.82	<0.001	19.9	8.93	<0.001	7.1	12.68	<0.001	39.1	14.03	<0.001
	SnW	0.419	21.6	95.04	<0.001	64.3	70.79	<0.001	2.5	11.02	<0.001	11.6	10.19	<0.001
	GRL2	0.534	45.1	192.57	<0.001	33.9	36.15	<0.001	0.7	2.81	0.094	20.4	17.41	<0.001
	AL	0.527	51.2	141.28	<0.001	37.2	25.65	<0.001	3.9	10.79	0.001	7.6	4.21	<0.001
Defense	FSL	0.608	55.9	511.29	<0.001	24.7	56.41	<0.001	2.0	17.97	<0.001	17.5	32.01	<0.001
	DSL	0.507	58.1	517.04	<0.001	21.6	48.14	<0.001	3.5	31.35	<0.001	16.8	29.90	<0.001
	PSL	0.784	62.5	773.23	<0.001	20.8	64.49	<0.001	0.4	4.63	0.032	16.3	40.27	<0.001
	PGL	0.224	63.2	1835.81	<0.001	22.4	162.75	<0.001	3.3	95.75	<0.001	11.1	64.58	<0.001
Body	BD1	0.356	8.4	16.88	<0.001	41.8	21.04	<0.001	2.0	4.02	0.045	47.9	19.30	<0.001
	BD2	0.343	21.5	32.74	<0.001	36.0	13.70	<0.001	1.5	2.22	0.136	41.0	12.46	<0.001
	CPL	0.094	9.3	53.45	<0.001	88.5	127.49	<0.001	0.0	0.02	0.901	2.2	2.59	0.025
	PGW	0.183	75.4	618.94	<0.001	20.2	41.55	<0.001	0.0	0.32	0.574	4.3	7.11	<0.001
	TLP	0.355	0.5	1.13	0.287	23.5	13.59	<0.001	11.0	25.46	<0.001	65.0	30.02	<0.001
	BLA	0.348	27.5	121.42	<0.001	36.6	40.35	<0.001	22.1	97.33	<0.001	13.8	12.15	<0.001
	BLD	0.257	40.1	167.44	<0.001	47.8	49.97	<0.001	5.9	24.57	<0.001	6.2	5.17	<0.001

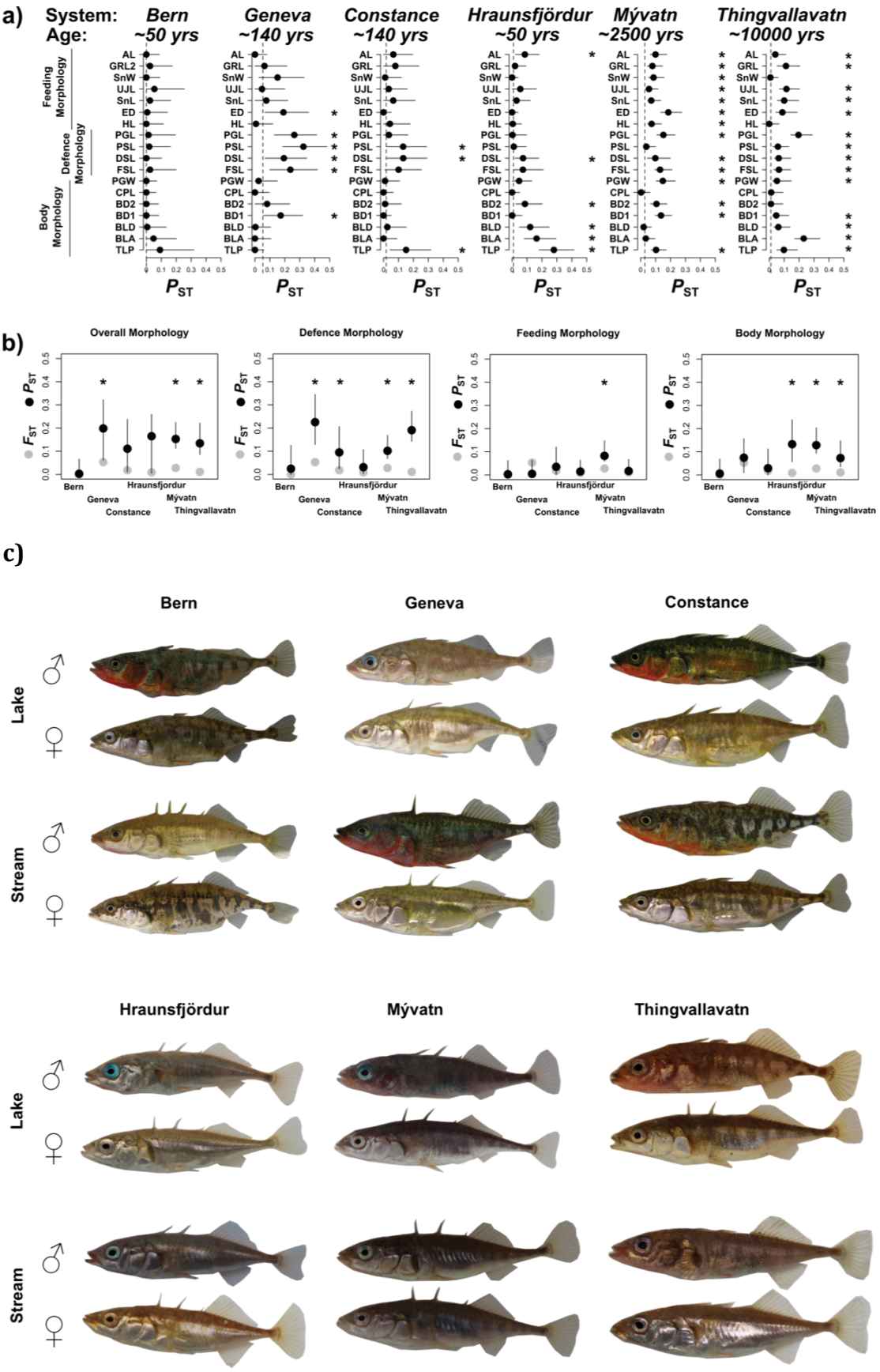

Figure 3: *Pairwise phenotypic divergence (P_{ST}) for each system in relation to the degree of genetic divergence (F_{ST}) either calculated for each trait separately (a) or*

for functionally distinct groups (b) with a graphical representation of the different ecotypes (c). Black dots indicate pairwise P_{ST} with their 95% confidence interval and F_{ST} values are given as either dashed lines (a) or grey dots (b). P_{ST} for functional groups is based on PC scores of the leading axis for either all traits combined, defence related traits, feeding related traits, body shape related traits (see text for details). Asterisks indicate cases where P_{ST} values are outside of the 95% confidence range of F_{ST} and hence significantly differentiated. For a description of each trait and its abbreviation see the main text and Figure S1. (c) Representative examples of the different stickleback ecotypes for each lake. For each lake and ecotype a male and a female are shown.

The angle θ between the leading axes of phenotypic evolution (p_{max}) between parapatric lake and stream populations based on all phenotypic traits differed significantly from zero in all cases except Thingvallavatn (Figure 4, Table 2). θ was highest in the two other Icelandic systems (Table 2), whose p_{max} were in addition significantly differentiated from all other freshwater systems in the pairwise comparisons (Table 3). θ between parapatric lake and stream populations within the Swiss systems was significantly different from zero too (Table 2) and followed similar overall trajectories (Table 3).

When traits were analyzed by functional categories, the angle θ between parapatric lake-stream p_{max} differed across traits and systems (Figure 4): For defense related traits, θ between the ecotypes was significantly differentiated only in Hraunsfjördur (Table 2). In terms of feeding related morphology the angle between the p_{max} of parapatric ecotypes was generally small (average θ: 9.3° ± 2.3° SD, Table 2) but differed significantly in Constance, Geneva and Mývatn with similar trends seen in Hraunsfjördur and Thingvallavatn (i.e. $0.05 < p < 0.1$, Figure 4). For body depth and swimming performance related traits, θ differed between parapatric ecotypes only in Constance (79.0°) and Geneva (74.4°). None of the angles between parapatric ecotypes were statistically correlated with the age of the system, the altitudinal difference or the geographical distance between the lake and the stream populations (all $p > 0.1$, results not shown).

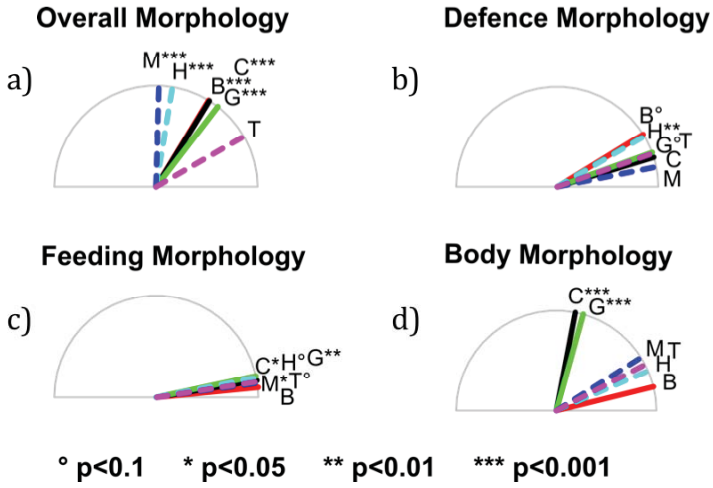

Figure 4: *Angles between the major axis of phenotypic variation in parapatric lake versus stream populations. Angles were calculated including either all phenotypic traits (a) or a subset of defense (b), feeding (c) or body shape / swimming performance (d) related traits. Letters indicate the respective system: B – Bern (red), C – Constance (black), G – Geneva (green), H – Hraunsfjördur (blue), M – Mývatn (dark blue), T – Thingvallavatn (pink). Dashed lines denote lake-stream systems from Iceland, solid lines systems from Switzerland.*

Table 2: *Angle between the leading eigenaxes (θ) of either the marine population and the overall system, stream or lake population only, as well as the angle between the parapatric lake-stream populations. The analysis was either performed combining all traits or separately for defense, feeding or body shape related traits. p values are based on 1000 bootstrap replicates with the one-tailed 97.5% confidence limit (CL) indicated. Significant p values (< 0.05) are given in bold, p values (<0.1 < p < 0.05) are in italic.*

	Marine vs. System			**Marine vs. Stream**			**Marine vs. Lake**			**Parapatric Lake-Stream**		
	θ (°)	***p***	***97.5% CL***	θ (°)	***p***	***97.5% CL***	θ (°)	***p***	***97.5% CL***	θ (°)	***p***	***97.5% CL***
All traits												
Bern	**63.97**	**<0.001**	**24.56**	**84.89**	**<0.001**	**4.66**	**52.10**	**<0.001**	**33.99**	**58.67**	**<0.001**	**29.67**
Geneva	**51.07**	**<0.001**	**34.47**	26.11	0.275	59.49	**56.77**	**<0.001**	**30.42**	**52.36**	**<0.001**	**34.42**
Constance	**68.27**	**<0.001**	**20.76**	**70.65**	**<0.001**	**18.35**	**58.68**	**<0.001**	**29.44**	**58.14**	**<0.001**	**30.79**
Hraunsfjördur	**53.47**	**<0.001**	**35.15**	**55.61**	**<0.001**	**31.15**	**55.97**	**<0.001**	**31.78**	**80.52**	**<0.001**	**8.55**
Mývatn	**64.22**	**<0.001**	**20.73**	38.89	0.134	48.87	**65.11**	**<0.001**	**18.51**	**88.23**	**<0.001**	**0.87**
Thingvallavatn	**75.37**	**<0.001**	**13.46**	**73.21**	**<0.001**	**15.69**	**69.72**	**<0.001**	**16.19**	29.88	0.243	50.52
Defense traits												
Bern	14.70	0.152	26.16	8.45	0.376	25.42	**39.34**	**0.016**	**36.00**	*31.69*	*0.058*	*46.52*
Geneva	4.28	0.538	21.97	5.67	0.548	29.68	14.48	0.147	27.16	*19.95*	*0.088*	*28.96*
Constance	*17.31*	*0.071*	*22.72*	14.04	0.151	29.49	27.41	0.147	50.16	17.10	0.298	59.16
Hraunsfjördur	13.21	0.208	27.32	**31.90**	**0.008**	**25.14**	2.02	0.921	38.75	**30.41**	**0.001**	**21.32**
Mývatn	**23.68**	**0.026**	**23.78**	7.16	0.517	35.50	*17.63*	*0.067*	*20.77*	11.43	0.203	27.26
Thingvallavatn	**19.40**	**0.047**	**21.25**	7.92	0.455	39.29	*18.84*	*0.054*	*20.98*	19.36	0.105	42.79
Feeding traits												
Bern	*5.56*	*0.062*	*6.80*	7.06	0.182	14.05	4.83	0.106	6.68	5.63	0.335	13.75
Geneva	*6.77*	*0.060*	*7.87*	4.81	0.289	8.48	**12.28**	**0.003**	**8.14**	**12.11**	**0.009**	**10.02**
Constance	**8.17**	**0.001**	**5.00**	**9.88**	**0.011**	**7.28**	**9.60**	**0.003**	**6.36**	**9.52**	**0.033**	**10.07**
Hraunsfjördur	**10.27**	**0.007**	**8.23**	*7.67*	*0.081*	*10.42*	**13.53**	**0.009**	**11.51**	*11.36*	*0.071*	*14.47*
Mývatn	**7.07**	**0.002**	**5.32**	**7.11**	**0.021**	**6.66**	**8.06**	**0.006**	**5.99**	**8.13**	**0.012**	**7.45**
Thingvallavatn	**6.04**	**0.03**	**6.15**	**10.01**	**0.034**	**10.73**	**6.79**	**0.007**	**5.90**	*8.83*	*0.055*	*10.23*
Body traits / Swimming performance												
Bern	**57.19**	**<0.001**	**30.84**	**55.25**	**<0.001**	**32.94**	**56.50**	**<0.001**	**31.93**	13.80	0.437	38.91
Geneva	27.16	0.237	55.11	20.27	0.359	54.41	**87.37**	**<0.001**	**1.24**	**74.39**	**<0.001**	**13.89**
Constance	39.88	0.114	47.41	34.49	0.183	52.13	**77.28**	**<0.001**	**11.80**	**79.02**	**<0.001**	**9.53**
Hraunsfjördur	31.51	0.174	52.51	*39.42*	*0.094*	*47.27*	36.68	0.113	49.47	23.51	0.299	58.68
Mývatn	**39.51**	**0.031**	**40.68**	22.23	0.583	65.40	**38.47**	**0.042**	**43.57**	32.12	0.323	55.53
Thingvallavatn	**51.10**	**<0.001**	**36.08**	**62.98**	**<0.001**	**25.71**	**39.41**	**0.035**	**42.75**	27.46	0.252	57.98

Comparing the p_{max} between the allopatric systems separately for different functional categories, none of the pairwise angles between allopatric drainage systems were significant for feeding and defense related traits (Table 3). This suggests that populations in all freshwater drainage systems followed similar phenotypic trajectories in these trait categories. For body depth and swimming performance related traits, the p_{max} of the Constance and Geneva system differed significantly from those in all other systems but not from each other (θ between Constance-Geneva: 4.6°, $p = 0.715$; Table 3).

Parallel adaptation trumps historical contingency late but not early in ecotype formation

The Mahalanobis distances showed overall consistent morphological differentiation between Swiss and Icelandic freshwater stickleback populations (Figure 2b). Despite evidence for parallel evolution of ecotypic divergence in Switzerland and Iceland, based on analyses of P_{ST} and the P matrix, all populations of young ecotype pairs, i.e. Hraunsfjördur in Iceland and all the Swiss systems, clustered by historical lineage rather than by ecotype. Hence rapid parallel adaptation did not trump historical contingency. Among the populations from Iceland, all freshwater populations were separated from the Marine population 1. In contrast to the clustering by lineage of the young ecotype pairs, populations from the two oldest lake systems, i.e. Mývatn and Thingvallavatn, clustered strongly by ecotype despite being genetically more strongly differentiated than the lineages with young ecotype pairs (Figure 2a). The second marine population was the sister clade to the stream clade.

Phenotypic divergence during the marine-freshwater transition

The angle θ between p_{max} of freshwater systems, combining both lake and stream populations, and the pooled marine populations differed significantly in all replicates when all traits were pooled together (average θ: 62.7° ± 9.1° SD; Figure 5, Table 2). When including only defense related phenotypic traits, θ between freshwater systems and the marine populations differed significantly only for Mývatn and Thingvallavatn. For feeding related traits, θ was relatively low (average θ: 7.3° ± 1.7° SD) but differed either significantly ($p < 0.05$) or marginally significantly ($0.05 < p < 0.1$) between freshwater populations and the marine populations. Finally, p_{max} for body shape and swimming performance related traits differed from the marine population only for Bern and Thingvallavatn.

When comparing p_{max} of stream populations only against the marine p_{max} using all traits combined, all but two stream populations (Geneva and Mývatn) had a p_{max} with a significant angle θ against that of the marine populations (Table 2). Using defense related phenotypic traits, none of the p_{max} of stream populations was significantly differentiated from the marine populations except for the Hraunsfjördur stream population. For feeding related traits, θ was relatively low (average θ: 7.8° ± 2.0° SD) with three stream populations being significantly differentiated from the marine populations (Constance, Mývatn, Thingvallavatn) and Hraunsfjördur marginally significantly differentiated from the marine populations. In body shape and swimming performance related traits, the p_{max} of only two stream populations (Bern and Thingvallavatn) differed

significantly from that of marine populations, and there was a trend for Hraunsfjördur stream fish.

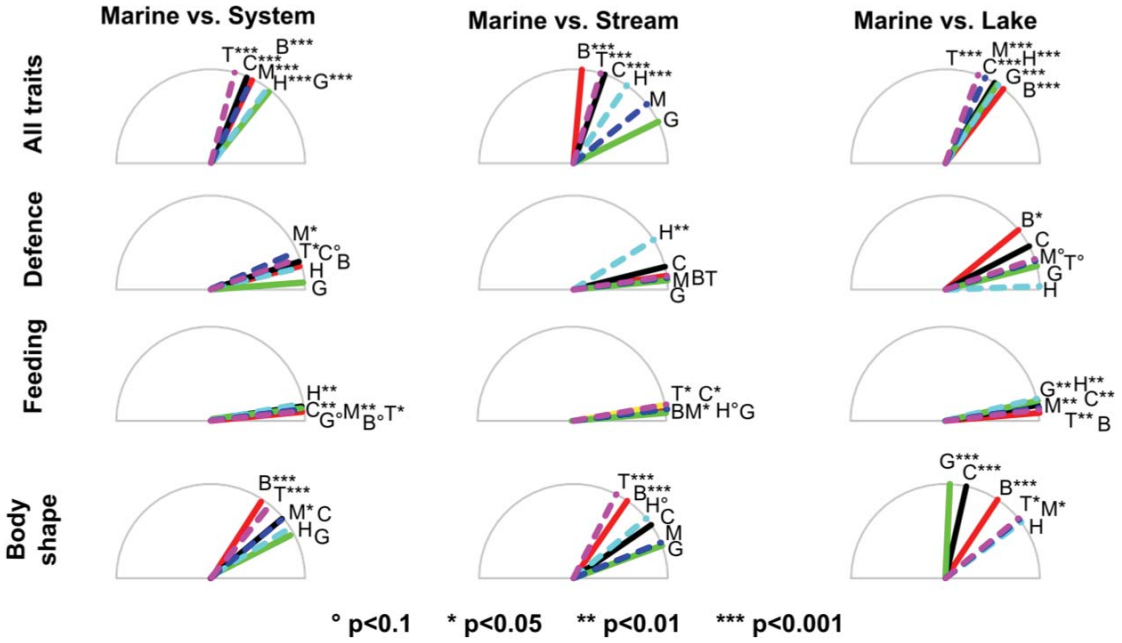

Figure 5: *Angles between the major axis of phenotypic variation between the marine population and (from left to right) freshwater populations (parapatric stream and lake populations for each system pooled), stream populations or lake populations only. Angles were calculated including either (from top to bottom) all phenotypic traits or a subset of defense, feeding or body shape / swimming performance related traits. For the overall divergence, indicated vectors are scaled according to the eigenvalue of the leading axis. Letters indicate the respective system: B – Bern (red), C – Constance (black), G – Geneva (green), H – Hraunsfjördur (blue), M – Mývatn (dark blue), T – Thingvallavatn (pink). Dashed lines denote lake-stream systems from Iceland, straight lines systems from Switzerland.*

The p_{max} for lake populations differed significantly from the combined marine populations in all cases when all traits were combined (average θ: 59.7° ± 6.5° SD). For defense related traits, p_{max} was only significantly differentiated from the marine p_{max} in Bern. In contrast, p_{max} for all lake populations except Bern differed in feeding related traits from the marine populations. In the latter case, the angles between the marine p_{max} and the freshwater lake p_{max} were relatively small (average θ: 9.2° ± 3.3° SD). Finally, all lake populations differed from the marine p_{max} except for Hraunsfjördur in body shape and swimming performance related traits with Swiss populations being furthermore more diverged than Icelandic ones (Table 3). In all but one case (marine vs. lake populations using all traits combined: $F_{1,4}$ = 15.9, p = 0.016), the observed angles θ between p_{max} of freshwater systems or populations and the p_{max} of the combined marine populations were not statistically correlated with the relative age of each freshwater system (all p > 0.1, results not shown).

Table 3: *Pairwise angle (θ) between the leading eigenaxes among freshwater systems (in degrees, lower triangular) with their corresponding p value (upper triangular). Axes were calculated using either all phenotypic traits, or a subset of traits related to defence, feeding or body shape respectively.*

All traits

	Bern	**Geneva**	**Constance**	**Hraunsfjördur**	**Mývatn**	**Thingvallavatn**
Bern	-	0.627	0.973	*0.078*	**0.007**	**0.025**
Geneva	6.31	-	0.635	**0.009**	**<0.001**	*0.081*
Constance	0.53	5.78	-	*0.052*	**0.001**	*0.055*
Hraunsfjördur	*21.85*	**28.16**	*22.38*	-	0.498	**<0.001**
Mývatn	**29.56**	**35.87**	**30.09**	7.72	-	**<0.001**
Thingvallavatn	**28.79**	*22.48*	*28.26*	**50.64**	**58.35**	-

Defense traits

	Bern	**Geneva**	**Constance**	**Hraunsfjördur**	**Mývatn**	**Thingvallavatn**
Bern	-	0.287	0.214	0.895	0.136	0.278
Geneva	11.75	-	0.737	0.232	0.308	0.939
Constance	14.59	2.85	-	0.194	0.596	0.774
Hraunsfjördur	1.28	10.47	13.32	-	*0.072*	0.257
Mývatn	20.26	8.52	5.67	*18.98*	-	0.304
Thingvallavatn	12.34	0.59	2.26	11.06	7.92	-

Feeding traits

	Bern	**Geneva**	**Constance**	**Hraunsfjördur**	**Mývatn**	**Thingvallavatn**
Bern	-	*0.059*	0.133	0.129	0.252	0.238
Geneva	*6.48*	-	0.395	0.809	0.259	0.279
Constance	3.89	2.59	-	0.597	0.576	0.765
Hraunsfjördur	5.73	0.75	1.84	-	0.403	0.447
Mývatn	2.49	3.99	1.40	3.24	-	0.738
Thingvallavatn	3.20	3.29	0.70	2.53	0.70	-

Body traits / Swimming performance

	Bern	**Geneva**	**Constance**	**Hraunsfjördur**	**Mývatn**	**Thingvallavatn**
Bern	-	**<0.001**	**<0.001**	0.406	0.284	0.260
Geneva	**60.59**	-	0.715	**<0.001**	**<0.001**	**<0.001**
Constance	**65.21**	4.62	-	**<0.001**	**<0.001**	**<0.001**
Hraunsfjördur	9.71	**50.88**	**55.50**	-	0.564	0.752
Mývatn	18.32	**42.27**	**46.90**	8.60	-	0.765
Thingvallavatn	13.66	**46.93**	**51.55**	3.95	4.65	-

Discussion

Parallel or convergent evolution of phenotypically similar ecotypes during ecological speciation depends on the genetic constraints, the selective environment as well as the time for evolution to act (Schluter & Nagel, 1995; Langerhans & DeWitt, 2004; Nosil *et al.*, 2009; Kaeuffer *et al.*, 2012; Nosil, 2012). Nonparallel phenotypic features may thus occur between independently evolved yet ecologically similar ecotypes and phenotypic convergence may be absent in evolutionary young systems. The time to reach convergence may furthermore depend on the dimensionality of traits that underlie ecotype formation, where parallelism or convergence may be rapidly reached if a single of few traits are involved (e.g. Schluter *et al.*, 2004). Multivariate integration on the other hand may need much more time to reach convergence (Young *et al.*, 2009; Kolbe *et al.*, 2011).

Analyzing the major phenotypic trajectory (p_{max}) of ecotypic divergence in stickleback populations of variable age, occupying contrasting lake and stream habitats, and their marine ancestors, we find that phenotypic evolution can proceed along parallel evolutionary trajectories. Depending on the trait combination, adaptation to freshwater from the ancestral marine population follows similar trajectories (Figure 5, Table 2) and subsequent parapatric ecotype formation can likewise evolve along shared trajectories (Figure 4, Table 3). Although we predicted an increase over time in the angle between the ancestral p_{max} and the p_{max} of each derived population, we found no support for this. Conversely the extent and parallelism of ecotype formation seems to be driven by historical contingency and the time for evolution to act (Figure 2 & 3, Table 1), where the phenotypically most distinct parapatric ecotypes in the two oldest lakes, Mývatn and Thingvallavatn, cluster together in the phenotypic tree, despite being genetically very distinct. Depending on the examined trait combinations, the observed patterns vary however between countries and systems and may reflect diverse evolutionary histories that can arise from different genetic and environmental effects (Eroukhmanoff *et al.*, 2009; Berner *et al.*, 2010; Kaeuffer *et al.*, 2012).

Parallel evolution of freshwater stickleback

The evolutionary transition between the marine and freshwater environment has been repeatedly studied in stickleback (Kristjánsson, 2005; Wund *et al.*, 2008; Berner et al., 2010b; Jones et al., 2012b; Voje *et al.*, 2013), where especially divergent habitat dependent selection seems to drive phenotypic differentiation (Barrett et al. 2008). Colonization of freshwater habitats requires adaptation to new selective regimes, including but not restricted to different predation and prey communities (Gross, 1978; Gross & Anderson, 1984; Reimchen, 1994). The selective regimes may however differ between distinct freshwater habitats (Gross, 1978; Gross & Anderson, 1984; Reimchen, 1994; Berner *et al.*, 2009; 2010b). Consequently the degree of evolutionary divergence from an ancestral-like marine phenotype may differ between distinct habitats and among phenotypic traits, depending on the selective regime, the evolutionary history of a population and the time for

evolution to act. We find that the three Icelandic freshwater systems and the Swiss clade derived independently from a marine ancestor (Figure 2a). Concomitantly, the derived freshwater p_{max} differs significantly from the ancestral marine one in all but two comparisons when all phenotypic traits were combined (Table 2, Figure 5).

When lake and stream habitats and distinct functional trait combinations were separately analyzed, patterns for p_{max} vary (Figure 5, Table 2), potentially driven by distinct habitat dependent selective regimes and evolutionary constraints. In contrast to the observed divergence in the overall p_{max}, anti-predator related phenotypic adaptation to freshwater proceeds mainly along a marine-like p_{max} for both lake and stream habitats. Predator communities are thought to differ though, where marine and freshwater lake populations experience a predation regime dominated by gape limited predators such as birds and piscivorous fish (Gross, 1978; Reimchen, 1992), which shifts to increased insect predation in freshwater streams (Reimchen, 1994; Marchinko, 2009). Invertebrate predation may however be negligible in Iceland (Lucek *et al*., 2012b) and empirical evidence for the role of invertebrate predators as a source of selection is mixed for Swiss populations (Zeller *et al*., 2012a, b). Our results nevertheless show that adaptation to very different habitats with potentially different predator communities follows a common ancestral evolutionary trajectory. The distinct predator communities are thought to represent different adaptive peaks (Vamosi, 2002), which, given our observed small angles θ, could be easily reached.

In stickleback, habitat dependent ecotype formation within freshwater stickleback is commonly associated with a diet shift and subsequently changes in trophic morphology. Whereas marine and many freshwater lake stickleback forage commonly on zooplankton, freshwater stream fish feed generally on benthic prey (Gross & Anderson, 1984; Berner, 2009; Lucek et al., 2012a). Evidence from Icelandic lake populations furthermore suggests that these can forage predominantly on benthic invertebrates too, depending on the respective substrate (Kristjánsson et al., 2002a). The relatively small but significant angles θ suggest that the marine-freshwater transition leads commonly to an evolution away from the marine p_{max} (Figure 5). However, the freshwater axes are only little yet significantly differentiated in most cases from the marine axis, which is especially true for lake dwelling populations (Table 2). This is consistent with prior findings in Canadian stickleback, where freshwater lake populations always evolved a p_{max} that is diverged from the marine population, involving a shift in gill raker lengths (Berner et al., 2010b).

Freshwater ecotypes in stickleback are also commonly diverged from the marine population in body shape and swimming related traits that are linked to different foraging strategies in open water habitats and streams (Hendry & Taylor, 2004; Reid & Peichel, 2010; Hendry *et al.*, 2011). Hence similar divergent selective regimes as for feeding related traits could have let to the observed pattern. However, here θ differs between the Swiss and Icelandic systems (Table 2), where Swiss populations are highly divergent from the marine p_{max}, implicating different historical contingencies or the absence of gene flow from the marine population. The wide range for θ for both the overall system and

stream sites could reflect different selection regimes within the stream sites due to environmental differences such as differences in the flow regimes (Steppan *et al.*, 2002; Ravinet et al., 2013b).

Although the marine-freshwater transition seems to follow relatively similar major phenotypic trajectories for different trait categories, the degree of phenotypic differentiation differs among systems (Figure 2b). This may reflect differences in the selection histories and historical contingencies between our studied systems as well as differences in the time for evolution to occur, which becomes apparent when the phenotypic and the genetic based tree are compared (Figure 2). In the genetic tree, the two oldest lakes Mývatn and Thingvallavatn form distinct genetic clusters with the longest branch lengths, whereas Swiss populations, albeit being genetically very distinct from each other, form together a separate branch. In contrast, in the phenotypic tree, ecotype specific clusters occur in the old lakes, whereas all populations of young ecotype pairs cluster in concordance with their genetic lineage. Lineage dependent phenotypic constraints may have consequently been retained in Switzerland as the Constance and Geneva systems were seeded about 140 years ago by genetically distinct freshwater lineages where the Bern system lies in a hybrid zone between different lineages (Lucek *et al.*, 2010). In contrast, Icelandic freshwater populations likely derive from a common marine population, where gene flow from the ancestral marine population may still be possible (Ólafsdóttir et al., 2007a, Figure 2a).

Historical contingency and parallelism of parapatric lake-stream divergence

Both the occurrence and the extent of parapatric divergence depend mainly on the underlying environmental and selective gradient as well as the time for evolution to act (Endler, 1977; Doebeli & Dieckmann, 2003; Nosil *et al.*, 2009). Consequently parallel evolutionary divergence is only expected when the selective regimes are very similar among systems (Kaeuffer *et al.*, 2012). The repeated formation of parapatric lake-stream freshwater stickleback systems has been proposed to provide such a case (Reimchen *et al.*, 1985; Thompson *et al.*, 1997; Hendry & Taylor, 2004; Berner *et al.*, 2009; Lucek *et al.*, 2013). However, recent studies suggest non-parallelisms in the realized divergence that occur both on smaller geographical scales (Kaeuffer *et al.*, 2012; Ravinet et al., 2013b) as well as between continents (Berner *et al.*, 2010). In the latter case, the authors suggested that genomic constraints could be responsible for the observed lower degree of divergence among Swiss populations and the evolutionary younger Atlantic stickleback lineage in general (Orti *et al.*, 1994), where only the Constance system showed similar levels of divergence as observed in Canadian systems (Berner *et al.*, 2010; Ravinet et al., 2013b, but see Lucek *et al.*, 2013). However, the underlying evolutionary trajectories have not been compared.

Our results indeed suggest that the evolution of parapatric lake-stream populations in stickleback can proceed along a common p_{max} as it is indicated by the non-significant angles for defense and feeding related traits (Table 3). For the other trait combinations, p_{max} rather differs among countries. However, despite

evolving along similar evolutionary trajectories, only a relatively small fraction of the overall phenotypic variation can be attributed to parallel habitat dependent differentiation (Table 1). A much larger fraction is explained by the system and habitat interaction accounting for the combined effect of system related historical contingency and ecotypic divergence (Langerhans & DeWitt, 2004; Eroukhmanoff *et al.*, 2009; Kaeuffer *et al.*, 2012). This is consistent with the pattern observed when contrasting the phenotypic and the genetic tree (Figure 2). It suggests that the evolutionary outcome in a given system may differ due to the lack of time for evolution to act or due to different historical contingencies as well as environmental differences among systems, which may slow down the emergence of similar phenotypes. The strongest case for parallelism occurred for anti-predator related morphology, with significant parapatric P_{ST} in four out of six cases (Figure 3), where the extent of divergence differs across freshwater systems and may reflect different predation regimes (Kaeuffer *et al.*, 2012). The dimensionality of parapatric phenotypic divergence seems to be generally increased in the two oldest lake systems (Figure 3), suggesting the importance of time for parapatric ecotype formation (Nosil & Sandoval, 2008).

Although we find that the evolution of parallel phenotypic divergence along parallel trajectories occurs quickly and becomes measurable within 50-100 years (Figure 3 & 4), phenotypic convergence needs much more time for parallelism to trump historical contingency (Figure 2). Consequently the time available for evolution is crucial for convergent ecotype formation (Nosil *et al.*, 2009; Nosil, 2012) and convergent evolution during adaptive radiations (Young *et al.*, 2009). Convergent phenotypic evolution seems to be furthermore associated with increased phenotypic integration, where habitat dependent parapatric divergence occurs in many more traits in evolutionary older systems (Figure 3). This is consistent with older adaptive radiations that show increased convergence in multivariate trait dimensions (Young *et al.*, 2009; Kolbe *et al.*, 2011).

In contrast to the observed phenotypic divergence and convergence, the degree of parapatric genetic differentiation is correlated with the parapatric environmental gradient rather than the evolutionary age of the system. Altitudinal gradients have similarly been found to explain the degree of parapatric genetic divergence in other freshwater systems (Ravinet *et al.*, 2013b) as well as between the marine and freshwater transition (Lucek *et al. unpublished data*) and may be linked to physical barriers restricting the potential for gene flow. Because the P_{ST}s for functional trait groups was not correlated with any environmental factor or F_{ST}, cases where phenotypic divergence was observed occurred likely through directional natural selection (i.e. $P_{ST} > F_{ST}$ (Merilä & Crnokrak, 2001)). In such cases, phenotypic divergence can be a consequence of divergent adaptation to different habitats, proceeding either along a common p_{max} or not. Anti-predator related traits show indications for directional selection in all lakes except Bern whereas feeding related traits are only diverged among Icelandic systems (Figure 3). This suggests that parapatric phenotypic lake-stream divergence is driven by habitat dependent selection related to different predation regimes (Reimchen, 1992; 1994; Marchinko &

Schluter, 2007) and diet shifts (Berner *et al.*, 2009; Lucek et al., 2012a; Kaeuffer *et al.*, 2012).

Rapid evolution versus plasticity

Although phenotypic divergence was greatest in the oldest lakes, the observed differentiation in $p_{\max}$ was not associated with our studied temporal gradient. Hence, plasticity may initially promote the colonization of freshwater habitats (Smith & Skúlason, 1996), by rapidly shifting the major phenotypic trajectory (Lande, 2009; Draghi & Whitlock, 2012). Marine stickleback are known to be phenotypically plastic, allowing them to adapt to different diets readily when colonizing new freshwater environments (Wund *et al.*, 2008). Plasticity can furthermore evolve in freshwater to initially promote a generalist life style where divergent selection may then lead to a canalization and the reduction in plasticity (Svanbäck & Schluter, 2012). This is congruent with theoretical predictions which imply that increased phenotypic plasticity can evolve rapidly in novel habitats to allow adaptation to a new optimum (Lande, 2009; Thibert-Plante & Hendry, 2011). Phenotypic trajectories however evolve theoretically quite fast over less generations than our youngest studied system (Lande, 2009; Draghi & Whitlock, 2012), consequently plastic trajectories are expected to align if populations experience similar underlying selective regimes.

On the other hand, recurrent selection from standing genetic variation in the marine population has repeatedly been suggested to drive adaptive phenotypic shifts between the marine-freshwater transition as well as between distinct freshwater habitats in stickleback (Deagle *et al.*, 2012; Jones et al., 2012b). This is especially true for anti-predator related phenotypic shifts, where selection drives phenotypic divergence in only a few generations (Bell *et al.*, 2004; Barrett et al. 2008; Schluter & Conte, 2009) and may similarly account for phenotypic divergence in other genetically determined traits such as gill rakers (Hermida *et al.*, 2002). Our observed divergence in these traits may therefore be a combined result of both plasticity and standing genetic variation (Wund *et al.*, 2008; Eroukhmanoff & Svensson, 2011). However, as indicated by the increased dimensionality of the parapatric phenotypic divergence in the two oldest lakes (Figure 3), evolution may need time to build up divergence for many of our studied traits.

Conclusions

Our results suggest that parapatric phenotypic divergence can evolve along a common evolutionary trajectory for some trait combinations, i.e. trophic morphology, but that the directionality of change in these traits may differ due to historical contingency or environmental effects. Whereas the evolution of the major phenotypic trajectory – p_{max} – of freshwater populations from an ancestral marine population seems to be independent of our studied temporal axis, both the extent and the dimensionality of parapatric ecotype formation depend on the available time for evolution to occur. Thus evolutionary changes towards novel

adaptive peaks may occur readily during ecotype formation and may be aided by phenotypic plasticity, yet convergent phenotypic evolution needs time to overcome contingency.

Acknowledgment

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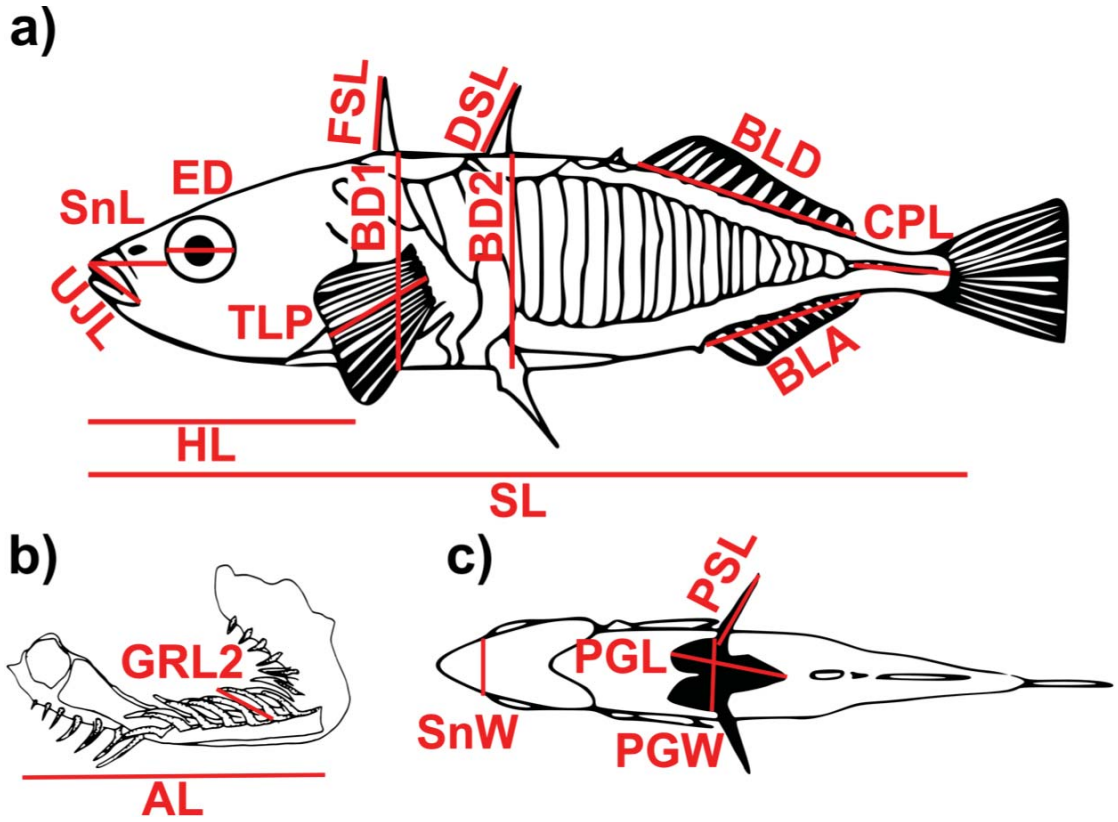

Figure S1: *Summary of all linear measurements used in this study that were either obtained on the left side a), the gill arch b) or from the ventral side of each individuals. These measurements can be categorized to belong to either anti-predator defense (FSL - length of the first dorsal spine; DSL - length of the second dorsal spine; PSL - length of the pelvic spine; PGL - length of the pelvic girdle), feeding ecology (HL - head length; UJL - upper jaw length; SnL - snout length; SnW - snout width; ED - eye diameter) or being linked to general body shape and swimming performance (SL - standard length; PGW - width of the pelvic girdle; BD1 -body depth measured after the first dorsal spine; BD2 - body depth measured after the second dorsal spine; CPL - caudal peduncle length; BLA - basal length of the anal fin; BLD - basal length of the dorsal fin; TLP - total length of the pelvic fin; see main text for details). Two feeding related traits were measured on the gill arch: the length of the second gill raker (GRL2) and the length of the lower gill arch (AL).*

Table S1: *Sampling sites with their respective geographic location, country of origin and the habitat with the number of individuals used for the phenotypic and the genetic data analysis as well as the coordinates where they were obtained.*

Site	Geographic Location	Country	Habitat	$N_{Phenotypic}$	$N_{Genotypic}$	Latitude	Longitude
BL	Lake Wohlen	Switzerland	Lake	30	30	46°57.594 N	7°21.872 E
BS	Chräbsbach	Switzerland	Stream	28	28	46°59.309 N	7°24.422 E
GL	Lake Geneva	Switzerland	Lake	40	30	46°31.289 N	6°34.417 E
GS	Rhone side channel	Switzerland	Stream	35	30	46°12.506 N	7°18.530 E
CL	Lake Constance	Switzerland	Lake	40	30	47°29.819 N	9°32.379 E
CS	Aubach	Switzerland	Stream	40	28	47°19.337 N	9°34.414 E
HL1	Hraunsfjördur	Iceland	Lake	40	40	64°55.901 N	23°01.657 W
HL2	Hraunsfjördur	Iceland	Lake	39	32	64°56.703 N	23°00.687 W
HS	Nameless stream	Iceland	Stream	37	30	64°55.132 N	23°00.888 W
ML1	Lake Mývatn	Iceland	Lake	30	35	65°37.997 N	16°37.398 W
ML2	Lake Mývatn	Iceland	Lake	30	28	65°37.665 N	16°55.231 W
ML3	Lake Mývatn	Iceland	Lake	30	33	65°36.867 N	17°03.501 W
ML4	Lake Mývatn	Iceland	Lake	39	38	65°36.431 N	16°57.861 W
ML5	Lake Mývatn	Iceland	Lake	41	38	65°37.295 N	16°57.024 W
ML6	Lake Mývatn	Iceland	Lake	30	30	65°38.538 N	16°55.662 W
MS1	Kráká	Iceland	Stream	31	30	65°32.169 N	17°03.664 W
MS2	Kráká	Iceland	Stream	31	0	65°34.298 N	17°04.330 W
MS3	Grænilækur	Iceland	Stream	25	0	65°33.691 N	16°59.992 W
TL1	Lake Thingvallavatn	Iceland	Lake	22	34	64°09.008 N	21°02.753 W
TL2	Lake Thingvallavatn	Iceland	Lake	30	30	64°14.646 N	21°05.390 W
TL3	Lake Thingvallavatn	Iceland	Lake	30	17	64°10.004 N	21°05.490 W
TL4	Lake Thingvallavatn	Iceland	Lake	57	50	64°11.173 N	21°05.227 W
TL5	Lake Thingvallavatn	Iceland	Lake	17	17	64°07.583 N	21°10.028 W
TS	Villingavatnsá	Iceland	Stream	39	30	64°06.452 N	21°05.804 W
Marine 1	Snæfellsnes	Iceland	Marine	45	39	65°02.659 N	22°27.476 W
Marine 2	Skagi	Iceland	Marine	62	0	66°06.655 N	20°05.656 W

Supplementary methods

Genomic DNA was extracted from fin tissue sample using a 10% Chelex solution, following the manufacturers protocol (Biorad, California, USA). 10 microsatellites were amplified in a single multiplexing set (Table S2).

The polymerase chain reaction (PCR) reactions consisted of 5 µl Qiagen Multiplexing Solution (Qiagen, Switzerland), 0.95 µl primer mix (Table S2), 3.05 µl dH_2O and 1 µl DNA per reaction. The PCR started with 15 min at 95°C followed by 26 cycles with 95°C for 30 seconds, 53°C for 90 seconds and 72°C for 60 seconds with a final elongation at 60°C for 30 minutes. PCR products were 1:10 diluted and visualized on a ABI 3130XL (Applied Biosystems, USA) following the manufacturers instruction. Alleles were scored using GENEMAPPER v4.0 (Applied Biosystems, USA).

Table S2: *Microsatellites used in this study with their fluorescent and concentration used. Primers and the position in the genome, i.e. linkage group, were obtained from Raeymaekers et al., 2007.*

Marker	QTL	Fluorescent	µl per reaction [10 µM]
Gaest66		Blue	0.1
STN30		Blue	0.1
STN96	2nd spine length	Blue	0.2
STN173		Green	0.05
STN196		Green	0.1
STN130	2nd spine length	Black	0.05
STN174		Black	0.1
STN185		Red	0.1
STN70		Green	0.1
STN26	1st spine length	Red	0.05

Chapter 7

Repeated intralacustrine radiation in Icelandic sticklebacks: ecosystem size predicts how far speciation goes

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Manuscript

Abstract

Ecological speciation is thought to proceed along a continuum from weak to strong adaptive divergence and reproductive isolation. Several factors that vary in time and space may facilitate or constrain the succession along this continuum. Comparative analyses using replicated events of speciation at variable stages in the continuum are valuable to learn about the role of these factors. Using unbiased sampling and a novel clustering method, we estimated the number of distinct phenotypic modes in threespine stickleback populations from nine lakes and one marine population in Iceland, for some of which evidence for ecological speciation had previously been demonstrated. Using the inferred number of phenotypic modes, genetic differentiation from the marine population, and physical lake and landscape variables, we ask if ecological opportunity, and isolation, respectively gene flow from the ancestral gene pool in the Sea, can help predict the occurrence and the extent of phenotypic diversification and ecological speciation within lakes. We find intralacustrine phenotypic diversification to be the rule rather than the exception. It happened in all but the youngest population. We also find multiple phenotypic clusters in many (5 or 6 of 9) lakes, with phenotypic traits of known ecological relevance differentiating among groups. Our genetic data imply that eco-phenotypic diversification has occurred in parallel in the different lakes, with indications of non-random mating, inferred from neutral genetic markers, in four out of nine studied lakes and indications of reproductive isolation between phenotypic clusters in two. Although neither the phenotypic variation nor the number of phenotypic modes in lakes were associated with any of our environmental variables, the dimensionality of phenotypic differentiation between ecotypes was significantly positively related to ecosystem size, and reproductive isolation was only found in the largest lakes where phenotypic differentiation was highly dimensional.

Introduction

Ecological speciation is thought to proceed along an evolutionary continuum from intraspecific variation with a single phenotypic and genotypic mode to bimodal distributions of phenotypic or genetic clusters with varying levels of reproductive isolation, and eventually two discrete and fully isolated species (Seehausen et al., 2008a; Hendry, 2009; Seehausen, 2009; Nosil *et al.*, 2009; Nosil, 2012). The progression from one to two species can be interrupted and reversed and consequently speciation may remain incomplete for a considerable amount of time. If this process operates in spatially structured metapopulations, variation in the degree of progression and reversal along the continuum is expected to result in pairs of ecologically differentiated populations at different stages (Seehausen, 2009; Nosil *et al.*, 2009). Although studies on the different stages along a speciation continuum within the same taxon have recently emerged (Nosil, 2012; Feder *et al.*, 2012), including examples from invertebrates (*Timema* walking-stick insects (Nosil & Sandoval, 2008), pea aphids (Peccoud *et al.*, 2009), *Heliconius* butterflies (Nadeau *et al.*, 2013)) and fishes (*Pundamilia* cichlids (Seehausen et al., 2008a), threespine stickleback (Hendry *et al.*, 2009)), the comparative investigation of the very early stages of the speciation continuum are yet rare. Some of these examine replicated cases of intraspecific diversification and usually study allo- or parapatric populations exposed to strongly contrasting environments (Langerhans *et al.*, 2007; Berner *et al.*, 2009; Lucek *et al.*, 2013) but relatively few sympatric examples exist (e.g. Snowberg & Bolnick, 2008; Kapralova *et al.*, 2011; Woods *et al.*, 2013).

At the very early stage of the diversification process, phenotypic variation may appear as a unimodal distribution without distinct phenotypic clustering (Doebeli & Dieckmann, 2000; Hendry *et al.*, 2009 but see Smith & Skúlason, 1996; Smith *et al.*, 1997). Diversification may proceed by divergent adaptation towards distinct peaks on the adaptive landscape, which can lead to the emergence of phenotypically differentiated clusters and a multimodal distribution of adaptive variation (Wright, 1932; Gavrilets, 2004; Leimar *et al.*, 2008). Such diversification may evolve either through phenotypic plasticity (West-Eberhard, 2003), the evolution of discrete polymorphisms coded by a major gene with dominance (Smith *et al.*, 1997) or through ecological speciation (Nosil, 2012). Divergence is initially relatively weak, but may increase over time through the combined action of divergent natural selection and declining rates of gene flow (Schluter, 2009). Subsequent genotypic discontinuity can arise or be strengthened if the phenotypes under divergent ecological selection were also under divergent sexual selection, or mediate behavioral reproductive isolation in other ways (Maan & Seehausen, 2011; Thibert-Plante & Gavrilets, 2013). These processes, acting on their own or in concert may eventually lead to the completion of ecological speciation (Nosil *et al.*, 2009; Nosil, 2012). The ecological theory of adaptive radiation, i.e. the rapid proliferation of a single ancestral lineage into ecologically differentiated species through release from inter- and the action of intraspecific competition, suggests that ecological speciation is facilitated in relatively isolated places where the isolation-constrained process of community assembly makes it that early colonists experience release from interspecific competition, creating the ecological opportunity that facilitates intraspecific niche expansion and building of

phenotypic variation (Yoder *et al.*, 2010). Isolation, on the other hand, though may slow the building of the intraspecific genetic variation that is required for niche expansion and may make the rate of diversification dependent of mutation and standing genetic variation in the original colonists. Gene flow from outside the isolated population may facilitate the origin of intraspecific variation, but may impede the emergence of reproductively isolated clusters (Seehausen et al., 2008b; Abbott *et al.*, 2013). While the relationship between isolation and radiation has received consideration at macroecological and macroevolutionary scales (MacArthur & Wilson, 1967; Losos *et al.*, 2009), it has gained much less attention at a microevolutionary scale. Here we study such a case, where we relate the degree of genetic and phenotypic diversification within several derived freshwater lake populations of threespine sticklebacks with measures of geographic isolation as well as differentiation between these lake populations and their marine ancestors.

Classical argumentation and theoretical models indicate that the number of species that evolve during adaptive radiation, increases with the number of potential available niches (Simpson, 1953; Schluter, 2000; Gavrilets & Vose, 2005). Although positive relationships between species diversity and habitat diversity are generally widespread (Ricklefs & Lovette, 1999), very few studies exist that quantify this relationship specifically for adaptive radiations (e.g Wagner *et al. submitted*). Area or total habitat size is a commonly used proxy to assess niche diversity (Gavrilets & Losos, 2009).

The threespine stickleback (*Gasterosteus aculeatus*) species complex is an ideal system to study the very early stages of adaptive radiation. Stickleback have repeatedly colonized freshwater systems and adapted to different habitats throughout the northern hemisphere, following the last glacial retreat (McKinnon & Rundle, 2002). In many cases, they have diverged ecologically from an ancestral marine species to different degrees, forming phenotypically distinct freshwater populations and species (McKinnon & Rundle, 2002; Snowberg & Bolnick, 2008; Hendry *et al.*, 2009). In quite a few cases these freshwater stickleback have further differentiated into distinct stream and lake ecotypes (Reimchen *et al.*, 1985; Kaeuffer *et al.*, 2012; Lucek *et al.*, 2013; Ravinet *et al.*, 2013a; b). On the contrary, intralacustrine radiations into distinctly adapted morphs or species within a given lake have been very rare (McKinnon & Rundle, 2002). This process has been especially well investigated in Canadian coastal lakes some of which contain two distinct species, feeding predominantly on benthic or on limnetic food, and the work on these systems has become foundational work in ecological speciation (Schluter & McPhail, 1992; Rundle *et al.*, 2000). The only other known cases of intralacustrine ecological speciation are described from Iceland (Kristjánsson *et al.*, 2002a; Ólafsdóttir *et al.*, 2006), where evidence suggests intralacustrine radiation into substrate-associated morphs in each of six lakes (Jonsson, 2002; Kristjánsson et al., 2002a; b). Because all of these lakes were colonized from very similar marine populations at some time after the last glacial maximum, but they vary in age and in their extent of subsequent isolation from the Sea, replicated lacustrine stickleback populations are a useful system to study the effects of isolation on the early stage of adaptive radiation.

Studies of ecological speciation in Iceland have focused on the two largest lakes, Mývatn and Thingvallavatn (Kristjánsson *et al.,* 2002a; Ólafsdóttir et al., 2007a; Ólafsdóttir & Snorrason, 2009; Millet *et al.*, 2013), but differentiation may occur more commonly among Icelandic lakes (Jonsson, 2002; Kristjánsson et al., 2002a; Ólafsdóttir *et al*., 2007c). In the two well-studied lakes, stickleback have been suggested to form phenotypically differentiated substrate-associated morphs: a lava type, a mud type and - in Thingvallavatn – additionally a deep water dwelling type that forages in *Nitella* algae meadows growing on mud substrate at water depths between 10-20 meters depth (Sandlund *et al.*, 1992b; Ólafsdóttir *et al.*, 2007a). The morphs are distinct in terms of antipredator defense traits as well as in their feeding habits (Kristjánsson *et al*., 2002a). Furthermore, positive assortative mating between the nitella and lava morph has been observed in laboratory experiments (Ólafsdóttir *et al.*, 2006). The morphs of Thingvallavatn evolved since the retreat of the Ice sheets about 8000 years ago (Sandlund, et al., 1992a). Some other lakes though are much younger. Mývatn and its stickleback population are only about 2500 years old (Einarsson *et al.*, 2004), and Hraunsfjördur was only colonized by stickleback as recently as in 1987 (Kristjánsson et al., 2002b).

We have sampled nine Icelandic lakes. We first test for a relationship between the extent of genetic and phenotypic divergence of lake stickleback from the ancestral marine population, and of the extent of phenotyic diversification within these lakes. We then test for the existence of distinct phenotypic clusters, predicting a larger number of divergent phenotypes and stronger phenotypic differentiation in larger and more heterogeneous lakes, where potentially more and more distinct ecological niches might be available.

Material and Methods

Sampling and data collection

In order to assess the effects of isolation and other environmental factors on the potential for within-lake stickleback diversification, nine Icelandic lakes were selected that cover a wide range of environmental gradients, most notably distance from the sea, elevation above sea and surface area (Figure 1, Table 1). For four of these lakes differentiated coexisting phenotypes have been described that are related to different substrates: Thingvallavatn with three (Kristjánsson *et al.,* 2002a; Ólafsdóttir *et al.*, 2007a), Mývatn, Galtaból and Frostastavatn with two phenotypically distinct morphs each (Jonsson, 2002; Kristjánsson et al., 2002a). Hraunsfjördur is a lagoon that got landlocked in 1987, where phenotypic and genetic differentiation between populations inhabiting different substrates has been documented (Kristjánsson *et al.*, 2002b; Ólafsdóttir *et al.*, 2007b). In addition, a marine population was sampled representing the presumed ancestral state.

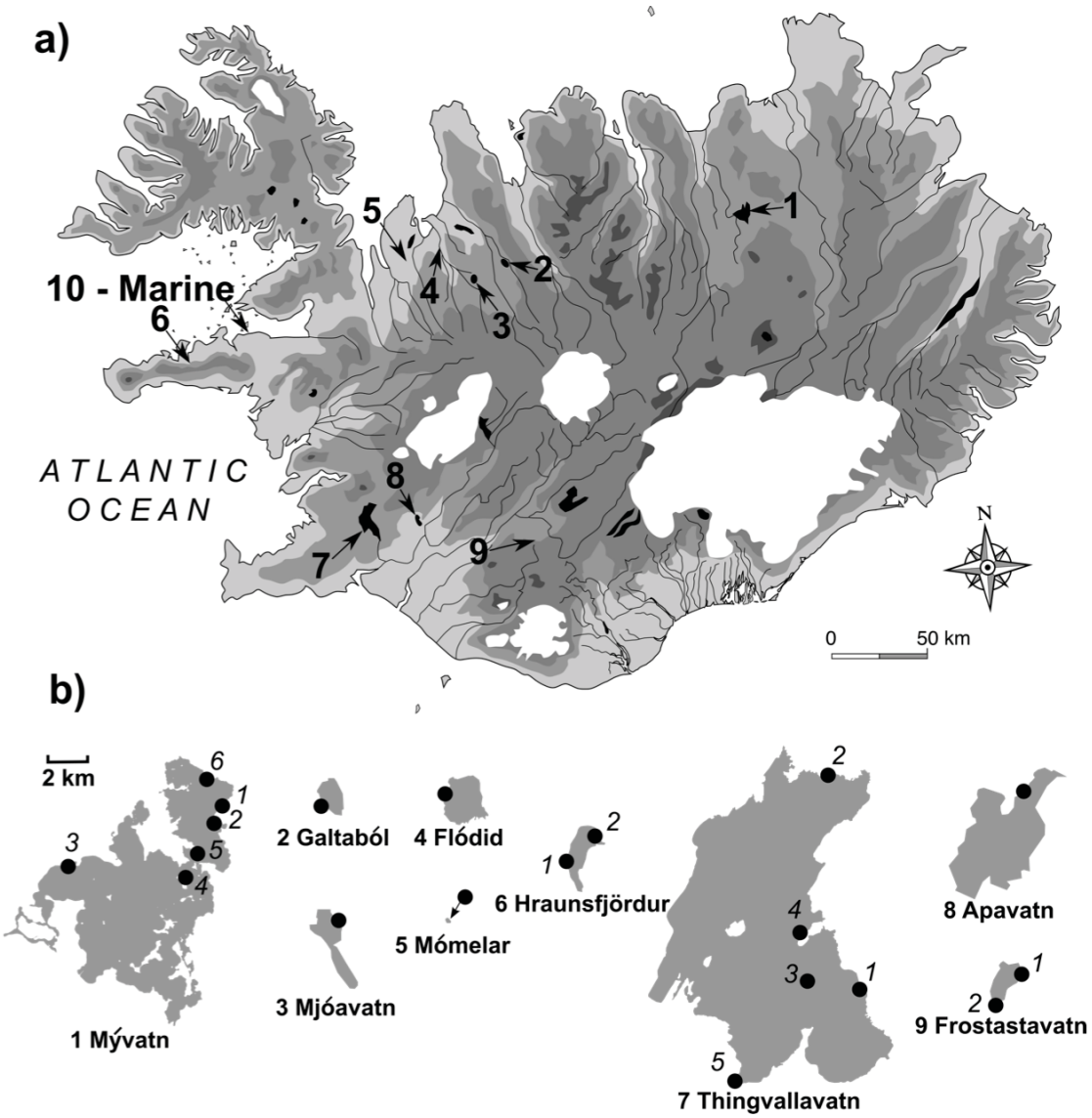

Figure 1: *a) Map of Iceland with sampled lakes indicated (modified from WIKIMEDIA © 2011). b) Outline of sampled lakes drawn to the same scale (modified from OPENSTREETMAP PROJECT © 2011). Black dots indicate the site where sticklebacks were sampled. Numbers in italic refer to the distinct sampling sites given in Table 1.*

Threespine stickleback were sampled from 21 locations among the nine lakes between August and September 2010 (Table 1) using minnow traps and by hand netting. All fish were humanely sacrificed with an overdose of clove oil and stored in ethanol. In addition, a fin clip was taken for further genetic analyses. The number of sampling locations within a single lake ranged from 1-6 depending on the size of the lake and the number of previously described distinct phenotypes. For lakes with a single sampling location, traps were placed such as to capture all available substrates. In such cases, individuals from all traps were pooled. Diversity of substrate types was inspected by eye and qualitatively recorded. For lakes where morphological differentiation had previously been studied, the established sampling locations were included in our sampling (Kristjánsson *et al.*, 2002a; Kristjánsson *et al.*, 2004). Sample size per site ranged from 17 to 71 individuals (mean: 40 ± 15 SD) with a total of 845 individuals analyzed.

Sixteen linear morphological traits, known to be associated with ecological divergence (see Reimchen *et al.*, 1985; Schluter & McPhail, 1992; Kristjánsson *et al.*, 2002a; Mori & Takamura, 2004; Berner *et al.*, 2008 and references therein) were measured to the nearest 0.01 mm using a digital caliper. These traits were either related to anti-predator defense (length of the first dorsal spine; length of the second dorsal spine; length of the pelvic spine; length of the pelvic girdle), feeding ecology (head length; upper jaw length; snout length; snout width; eye diameter) or to general body shape (Mori & Takamura, 2004) (standard length; width of the pelvic girdle; body depth 1 measured after the first dorsal spine; body depth 2 measured after the second dorsal spine; caudal peduncle length; basal length of the anal fin; basal length of the dorsal fin; total length of the pelvic fin). In addition, the length of the second gill raker, as counted from the joint of the dorsal arch bone of the first gill arch, and the length of the lower gill arch were measured using a micrometer mounted on a dissection microscope. Both measurements on the gill arch are related to feeding ecology (Berner *et al.*, 2008). Because all traits were significantly correlated with standard length (results not shown), a size correction was applied using the residuals of a regression of each trait against SL for each lake separately to remove potential differences in allometry between lakes. Additionally, both sagittal otoliths, calcium carbonate structures in the inner ear that show seasonal rings, were extracted from each individual. Winter rings were counted at 40x magnification using a microscope to estimate the age of each individual (Zeller et al., 2012a).

Genetic analysis

DNA for all individuals was extracted using a 10% Chelex solution, following the manufacturers protocol (Biorad, California, USA). Nine microsatellite markers (Gaest66, Stn26, Stn30, Stn96, Stn130, Stn173, Stn174, Stn185 and Stn196) were amplified in one multiplex kit following Raeymaekers *et al.* (2007). Three of these markers (Stn26, Stn96 and Stn130) have been shown to be associated with known QTLs for spine lengths (Peichel *et al.*, 2001). Consequently these markers are predicted to lead to genetic substructure if they are linked to a phenotype under disruptive selection in contrast to the neutral markers. A detailed description of each marker together with the PCR and multiplexing protocols are available in the supplementary methods. Alleles were visualized on an ABI 3130XL and scored with GENEMAPPER 4.0 (Applied Biosystems, Switzerland). Sex of each individual was determined using a molecular marker (*Idh*) yielding either one or two bands (separated by 30 bp) in females and males respectively (Peichel *et al.*, 2004). Here, PCR conditions followed Peichel *et al.* (2004) and PCR products were analyzed on a 1.5 % agarose gel, where genotypes were scored by eye.

Table 1: *Summary table for all sampling sites: distance from the sea, surface area, maximal lake depth and altitude (meters above sea level), lake position, sampling site ID with its associated substrate and sample size for each sex (N).*

Lake	Distance from the sea [km]	Surface [km²]	Max depth [m]	Elevation (masl)	Latitude (N)	Longitude (W)	ID	Substrate	$N_{females}$	N_{males}
Apavatn	71	13	2.5	64	64°11.70'	20°37.98'	1	lava / vegetation	30	11
Flódid	15	2.1	1.5	11	65°29.45'	20°21.59'	1	mud / vegetation	26	4
Frostastavatn	137	2.3	11	581	64°01.42'	19°02.50'	1	mud	21	40
							2	lava / vegetation	52	24
Galtaból	62	1.7	10	457	65°15.74'	19°44.47'	1	lava / sand	29	28
Hraunsfjördur	5	2.3	84	3	64°55.90'	23°01.66'	1	mud	20	20
							2	lava	27	12
Mjóavatn	59	3.3	1.1	453	65°15.66'	19°48.04'	1	lava / mud	32	29
Mómelar	64	0.03	2	142	65°25.29'	20°39.93'	1	lava / mud	25	9
Mývatn	23	37	4.5	286	65°37.99'	16°37.40'	1	mud	14	16
							2	mud	8	22
							3	lava	13	17
							4	mud / vegetation	25	13
							5	mud / vegetation	22	18
							6	lava /mud	18	12
Thingvallavatn	57	82	114	107	64°09.01'	21°02.75'	1	lava	15	7
							2	lava	10	20
							3	vegetation*	17	13
							4	mud / vegetation	13	44
							5	lava	8	9
Marine	-	-	-	-	65°02.66’	22°27.48’	1	sand	31	14

* Sampled offshore between 10-20m depth in *Nitella* sp. meadows

In total, 791 out of 845 individuals measured for morphological traits, were successfully genotyped, whereas amplification failed for 54 individuals, distributed over all lakes and sampling sites. Molecular sexing failed for seven individuals, which were omitted from all the analyses that required information on sex. Sex ratios differed among our samples from different lakes (Table 1) but the overall distribution of sex ratios did not differ from a normal distribution (Shapiro-Wilk test: $W = 0.948$, $p = 0.64$).

Heterozygosity, pairwise F_{ST} between each lake population (pooling all sample sites within a lake) and the marine population as well as the F_{ST} between identified distinct phenotypic modes within a lake were then calculated using GENODIVE 2.0 (Meirmans & Van Tienderen, 2004). To further test if the observed patterns could be driven by putatively QTL linked markers, all F_{ST} calculations were additionally performed using either only putatively neutral or QTL linked markers. The obtained F_{ST} values were subsequently compared using paired *t*-tests. Pairwise F_{ST} did not differ between the putatively QTL linked markers and the neutral ones (pairwise F_{ST} between the marine population and each lake: paired $t_{1,8} = 0.03$, $p = 0.975$; pairwise F_{ST} between identified phenotypic modes: paired $t_{1,11} = 0.04$, $p = 0.972$). For all subsequent genetic analyses, all microsatellite markers were therefore pooled. Heterozygosity and the F_{ST} between each lake and the marine population were tested for a correlation with environmental variables (elevation, distance from the sea, lake surface area and maximal lake depth, Table 1) using linear models. In addition, the global F_{IS} value was estimated for each lake and for the marine population combining all samples using an AMOVA approach with 10'000 bootstrapping replicates to test for potential genetic substructure. Global F_{IS} was furthermore separately calculated for each sex for each population to test for potential genetic substructure that may be hidden in the combined data set. The genetic structuring within each lake, either for both sexes combined or for each sex separately, was further estimated using an admixture model implemented in STRUCTURE 2.3.3 (Falush *et al.*, 2007) with 30'000 burnin steps followed by 300'000 MCMC steps. The simulation was performed assuming 1-6 genetic clusters (K) with 10 replicates for each assumed K. The simulation was run separately for each lake and for the marine population, either both sexes pooled or separately for each sex when more than 20 individuals were available. The optimal number of genetic clusters was then determined by investigating the individual assignment plots, the log likelihood values of each run and their variation among runs for the same K. To establish the genetic relationship among the sampled sites and lakes, a genetic tree-like relationship was generated. The tree was based on Cavalli-Sforza distances of allelic frequencies using a neighbour-joining algorithm implemented in the program PHYLIP 3.69 (Felsenstein 2012). Significance was then estimated using 1000 bootstrapped resampling replicates. Finally, the pairwise F_{ST} among all sampling sites was calculated using GENODIVE with 1000 bootstrap replicates to assess significance.

Phenotypic diversification in lakes

Phenotypic diversity in each lake was estimated as the amount of morphospace occupied, defined as the size of the 95% confidence ellipsoid for all individuals of a particular lake on the first two principal component (PC) axes, using all size corrected traits together. This method makes the assumption that the total phenotypic variation within a system should be higher than the variation of the sampled individuals (Erwin, 2007). Relative ellipse size was calculated using a custom made script based on an implementation in the CAR package (Fox & Weisberg, 2011) in R 2.15.1 (R Core Team, 2012). Subsequently the estimates of morphospace for lake populations were first scaled by the highest value observed and then regressed against sample size, against lake characteristics (distance from the sea, surface area, elevation) and against observed heterozygosity using linear models.

To estimate the degree of overall phenotypic differentiation between the ancestral marine population and each freshwater lake, pairwise P_{ST}, an analog to Q_{ST}, which is based on phenotypic data of wild individuals (Spitze, 1993; Raeymaekers *et al.*, 2007), was estimated. P_{ST}s were based on the residuals (after regression on size) of the first PC axis of each lake population and the marine population. Calculations followed Kaeuffer *et al.* (2012), where P_{ST}s and their 95% confidence intervals were estimated using a resampling approach with 1000 replicates. Obtained P_{ST} values were regressed against distance of lake from the sea, lake surface area, lake elevation as well as the pairwise F_{ST} against the marine population using linear models.

Phenotypic clustering

In order to determine the minimum number of phenotypic modes or clusters present among stickleback in a given lake, the best clustering method for the morphological data was first determined. Four different methods were compared assuming 2-6 clusters in each case with CLVALID (Brock *et al.*, 2008): unweighted pair group method with arithmetic mean (UPGMA) based on Euclidean distances, minimization of within-class sum of squares for each cluster, divisive hierarchical algorithm and model based clustering using a maximum likelihood algorithm. CLVALID calculates seven different indices, measuring stability and internal validation for each model and assumed number of clusters. However, CLVALID does not allow to distinguish between a one or two clusters scenario, i.e. testing the null hypothesis of no phenotypic diversification. To determine the best method, a weighted rank aggregation was performed via a Cross-Entropy Monte Carlo algorithm using RANKAGGREG (Pihur *et al.*, 2009) for each lake separately using the indices calculated by CLVALID. Here, the UPGMA algorithm based on Euclidean distances performed best in seven out of ten cases and was within the top three among the others. Therefore UPGMA was used for all subsequent cluster analyses to allow for comparative analyses among lakes.

The number of statistical supported phenotypic clusters was then separately determined for each sex within a lake, using a dynamic hybrid tree cut (Langfelder *et al.*, 2008). In short, this method is based on a bottom up algorithm which first identifies preliminary clusters depending on a given minimal cluster size, the distance and distinctiveness of its neighboring objects and the

connectivity of branches within a cluster. In a second step, previously unassigned objects are tested for their proximity to the preliminary clusters and get either assigned or not (see Langfelder *et al.*, 2008 for details). Because this method is based on tree topology without prior assumptions on the number of inferred clusters, it provides an unbiased estimate for the number of clusters that are present in a given data set. For all lakes, the settings were as follow: minimal cluster size: 8 individuals, maximal scatter: 0.75, minimal gap size: 0.25, maximal distance for assignment: 0.90. The last three values relate to the fraction between the maximal node height observed in the underlying UPGMA tree and the 5th percentile of all node merging heights. The obtained clusters were stable unless extreme values were taken (results not shown). A minimal cluster size of eight was chosen to allow for subsequent statistical analyses on the identified clusters. This approach gives a conservative estimate of the minimum number of clusters, as clusters with only few individuals are omitted from subsequent analyses. The analysis was separately conducted for both sexes.

Identified intralacustrine clusters or modes were subsequently tested for an association with age based on otolith readings, size (standard length) as well as sampling sites within a lake or substrate type, where available (Table 1) using an ANOVA. Models were then compared using the Akaike information criterion (AIC). Statistical phenotypic differentiation among modes was furthermore tested with a MANOVA including all measured phenotypic traits using *mode* as a factor. Individual trait differentiation was further investigated with a *post hoc* ANOVA decomposition of the MANOVA analysis. Moreover the multivariate Mahalanobis distances between modes within a lake were calculated to assess the degree of divergence.

Results

The marine-freshwater transition

Both the observed heterozygosity within a lake and the pairwise F_{ST} of lake populations against the marine population were significantly correlated with elevation of lakes above sea level (heterozygosity: $R^2 = 0.811$, $F_{1,8} = 34.3$, $p < 0.001$; pairwise F_{ST}: $R^2 = 0.868$, $F_{1,7} = 45.5$, $p < 0.001$) but not with the distance of lakes from the sea (heterozygosity: $R^2 = 0.350$, $F_{1,8} = 4.3$, $p = 0.072$; pairwise F_{ST}: $R^2 = 0.278$ $F_{1,7} = 45.5$, $p = 0.145$). As predicted if upstream dispersal from the Sea to the lakes was constrained by elevational difference, heterozygosity decreased with increasing elevation of lakes. Simultaneously, the genetic differentiation from the marine population increased (Figure 2). Elevation and distance from the sea were positively correlated ($R^2 = 0.467$ $F_{1,8} = 8.6$, $p = 0.019$). Concomitantly, in the genetic population tree, geographically separated low elevation lakes (Apavatn, Flódid, Hraunsfjördur) are closely related to the marine population (Figure 3), where the pairwise F_{ST} among these low elevation lakes was generally low ($F_{ST} < 0.05$, Table S1). The upland lakes are all more strongly differentiated from the Sea ($F_{ST} > 0.15$) and pairwise F_{ST} among these is generally also high ($F_{ST} > 0.30$), with the exception of Galtaból and Mjóavatn ($F_{ST} = 0.146$). The phylogenetic relationship suggests that every lake represents an independent colonization event from the Sea except for three lakes Galtaból, Mjóavatn and Frostastavatn. These are the three upland lakes in our study,

suggesting a common colonization event, which might have occurred during the early phase of the isostatic adjustment of Iceland in the course of the Holocene (Le Breton *et al.*, 2010). This suggests that the stickleback populations in these lakes are very old populations, although we cannot rule out that Galtaból and Mjóavatn exchanged genes more recently, or in fact that one of them would have been colonized from the other one recently perhaps involving human stock translocation.

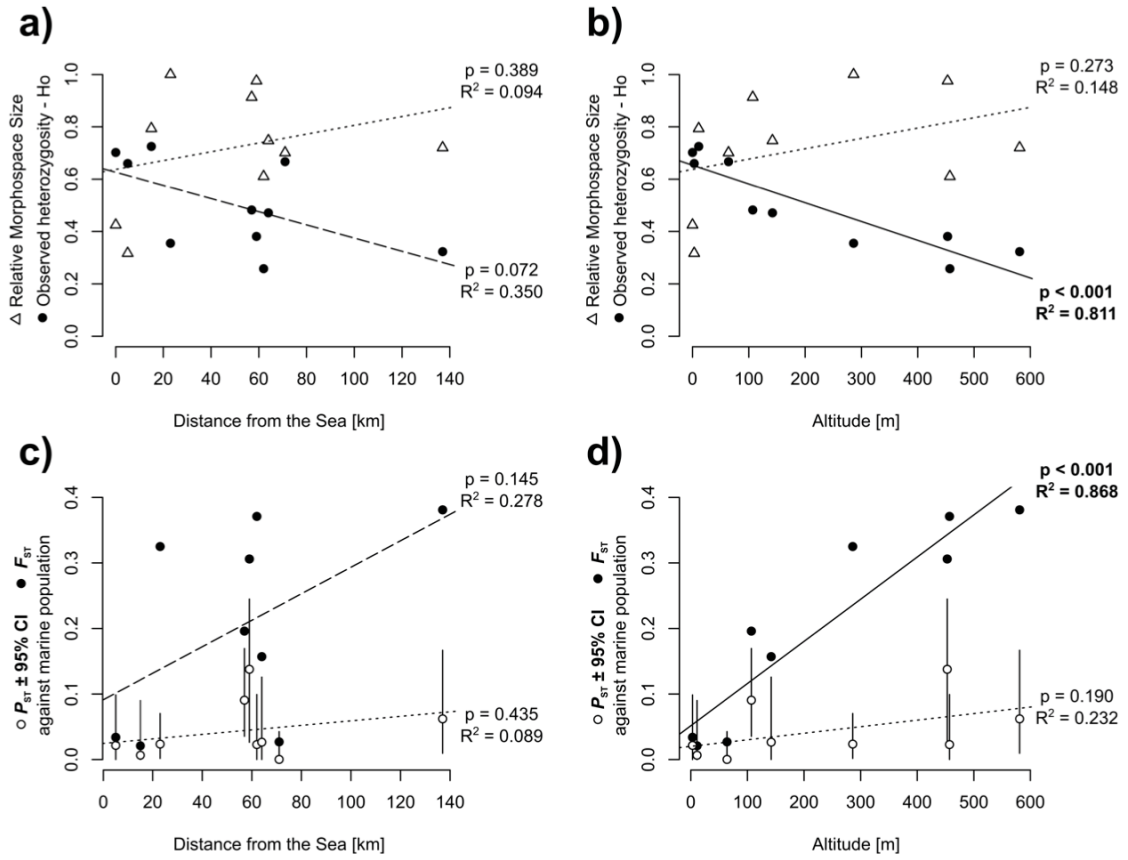

Figure 2: *Genetic and phenotypic variability, as well as genetic and phenotypic differentiation from the ancestral marine population, plotted against the distance from the Sea and against elevation (meters above sea level): a) Observed heterozygosity (black dots) and the relative size of occupied morphospace (triangles; see text for details) against the distance from the Sea, b) Observed heterozygosity (black dots) and the relative size of occupied morphospace (triangles) against elevation, c) Pairwise F_{ST} (black dots) and P_{ST} ± its 95% confidence interval – CI (white dots) of each lake against the marine population against the distance from the Sea, d) Pairwise F_{ST} and P_{ST} ± its 95% confidence interval – CI plotted against elevation. Regression coefficients and their significances are indicated, based on linear models.*

The first step towards ecological speciation: phenotypic diversification

The relative volume of morphospace occupied by stickleback within each lake did neither qualitatively differ when only females or males were analyzed, nor when the marine population was excluded from the PCA (results not shown). Therefore only the overall analysis including all individuals is shown (Figure 2). The first two PC axes accounted for 31.6% and 17.1% of the total variation respectively. The average scaled morphospace volume occupied by stickleback in each lake and the marine population on these first two PC axes was not correlated with sample size (R^2 = 0.193, $F_{1,8}$ = 1.9, p = 0.204). The Mývatn population showed the highest phenotypic variation, whereas the marine population and Hraunsfjördur, a 50 years old marine isolate, were the least variable, occupying 42.5% and 31.7% of the size of Mývatn respectively on PC1 and PC2.

As predicted by ecological speciation theory, the colonization of lakes from the Sea was associated with an increase in phenotypic diversity. Lakes had generally more diverse stickleback, i.e. their populations occupied a larger amount of the common morphospace, than the marine population (one sample *t*-test: $t_{1,8}$ = 4.70, p = 0.002). Morphospace volume of lake populations was not significantly associated with either distance from the sea (R^2 = 0.094, $F_{1,8}$ = 0.8, p = 0.389) nor elevation (R^2 = 0.148, $F_{1,8}$ = 1.4, p = 0.273; except that it was smaller in the marine population and the recent marine isolate than anywhere else (Figure 2). The estimated morphospace volumes for the lake stickleback populations were neither associated with lake surface area (R^2 = 0.210, $F_{1,7}$ = 1.9, p = 0.215) nor with maximum lake depth (R^2 = 0.071, $F_{1,7}$ = 0.5, p = 0.489). Morphospace volume was also not correlated with the observed heterozygosity at microsatellite markers (R^2 = 0.010, $F_{1,8}$ = 0.1, p = 0.795).

Phenotypic differentiation from the marine population, based on P_{ST} was strongest in Mjóavatn (P_{ST} = 0.138, 95%CI: 0.026-0.244, p = 0.012) and Thingvallavatn (P_{ST} = 0.091, 95%CI: 0.035-0.169, p = 0.013). P_{ST}s were not significantly associated with the distance from the sea (R^2 = 0.089, $F_{1,7}$ = 0.7, p = 0.435), nor with elevation (R^2 = 0.232, $F_{1,7}$ = 2.1, p = 0.190), lake surface area (R^2 = 0.077, $F_{1,7}$ = 0.6, p = 0.469) or lake depth (R^2 = 0.048, $F_{1,7}$ = 0.3, p = 0.573, Figure 2). P_{ST} and F_{ST} values between the lake populations and the marine population were furthermore not statistically correlated (R^2 = 0.224, $F_{1,7}$ = 2.0, p = 0.198), but F_{ST} values were on average significantly higher than their respective P_{ST} values (one sample paired *t*-test: $t_{1,8}$ = 3.53, p = 0.004), consistent with isolation from the Sea and relatively old age of many of the lake populations.

The second step towards ecological speciation: phenotypic differentiation

Taking the dynamic tree cut method together with respective lake specific MANOVA, our analyses identified distinct phenotypic clusters among females in five of nine lakes (Figure 3, Figure 4): Thingvallavatn, Myvatn, Mómelar, Frostastavatn, Apavatn and also in the Sea. No signal of bimodality was found in Hraunsfjördur or Mjóavatn, and two modes were found by the dynamic tree cut, but differences between these were not supported by MANOVA in lakes Flódid and Galtaból (Figure 4). Due to the relatively small sample sizes we had for males, only six lakes were available for a dynamic tree cut. No support for deviation

from unimodality in phenotype distribution was found for Galtaból and Hraunsfjördur, consistent with the analyses of females. For the other four lakes, Frostastavatn, Mjóavatn, Mývatn and Thingvallavatn bimodal distributions were found and the significant overall phenotypic differentiation between clusters was confirmed by MANOVA (Figure 4). Hence, significantly differentiated phenotypic groups of stickleback were found in Thingvallavatn, Myvatn, Frostastavatn, Mómelar, Apavatn and in the Sea, perhaps in Mjóavatn (only in males), but not in Hraunsfjördur, Flodid, or Galtaból. We did not find any indications of more than two phenotypic groups within either sex in any of the lakes. Therefore, we assume that the two clusters that we find in either sex in some lakes correspond to the same two ecotypes and not more.

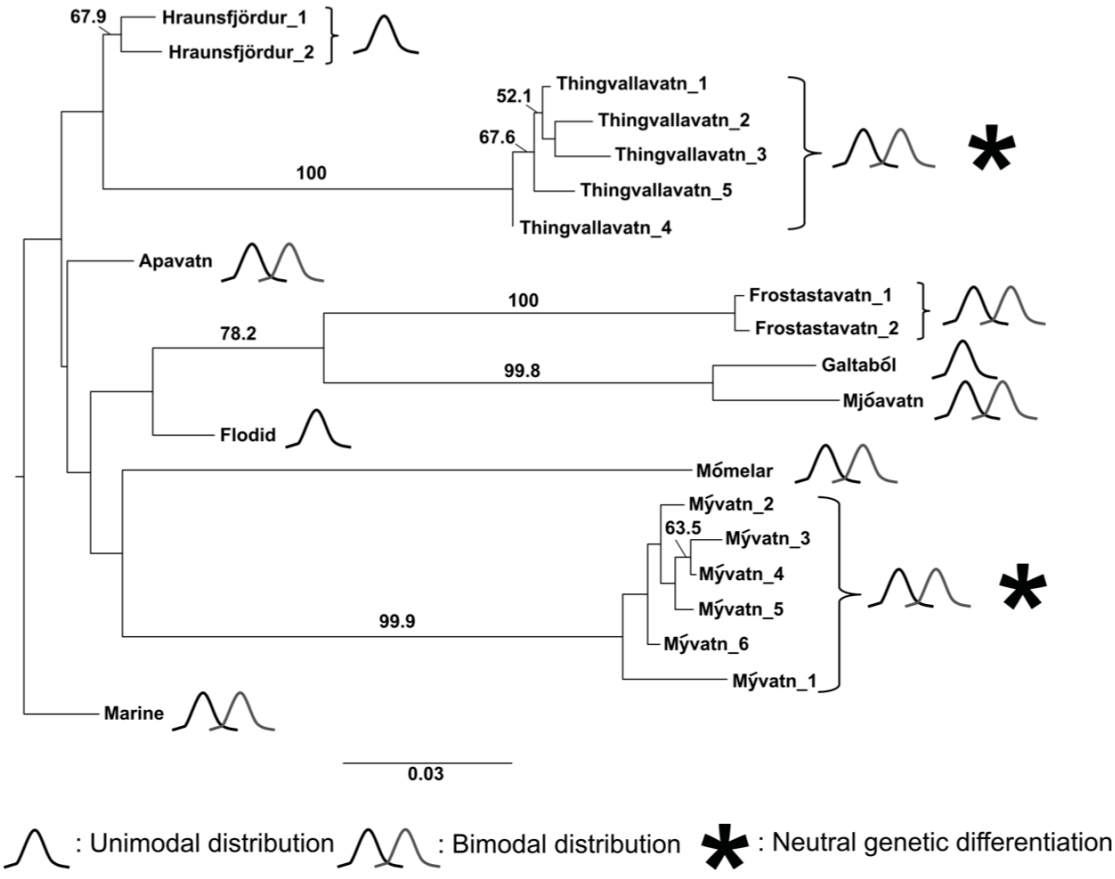

Figure 3: *Genetic relationships among Icelandic populations of lake stickleback with a marine population as outgroup. Neighbour-joining tree using Cavalli-Sforza distances amongst sampling sites included in this study (see Table 1), calculated from allele frequencies at 10 microsatellite loci. Numbers beside nodes indicate percent bootstrap support based on 1000 resampling replicates. Bootstrap values below 50% are not shown. Note that the deep part of this tree is effectively an unresolved polytomy, consistent with independent colonization from the sea for every lake except the three high altitude lakes Frostastavatn, Galtaból and Mjóavatn, suggestive of an earlier colonization event of these lakes during the early phase of the isostatic adjustment of Iceland during the melting of the Icelandic ice sheets (Breton et al. 2010). Symbols depicting bimodal distributions indicate cases, where two phenotypic modes have been identified that differ statistically from each*

other (see Figure 4). Asterisks indicate cases where neutral genetic differentiation was found between modes based on pairwise F_{ST} and STRUCTURE (see Table 1).

No associations were found between the number of phenotypic clusters in a lake (1 or 2) and either of the available environmental variables (elevation – females: $F_{1,7}$ = 0.0, p = 0.969, males: $F_{1,4}$ = 0.4, p = 0.575; distance from the sea – females: $F_{1,7}$ = 0.9, p = 0.386, males: $F_{1,4}$ = 0.8, p = 0.427; lake surface area – females: $F_{1,7}$ = 0.6, p = 0.478, males: $F_{1,4}$ = 1.1, p = 0.359; maximal lake depth – females: $F_{1,7}$ = 0.4, p = 0.558, males: $F_{1,4}$ = 0.1, p = 0.774).

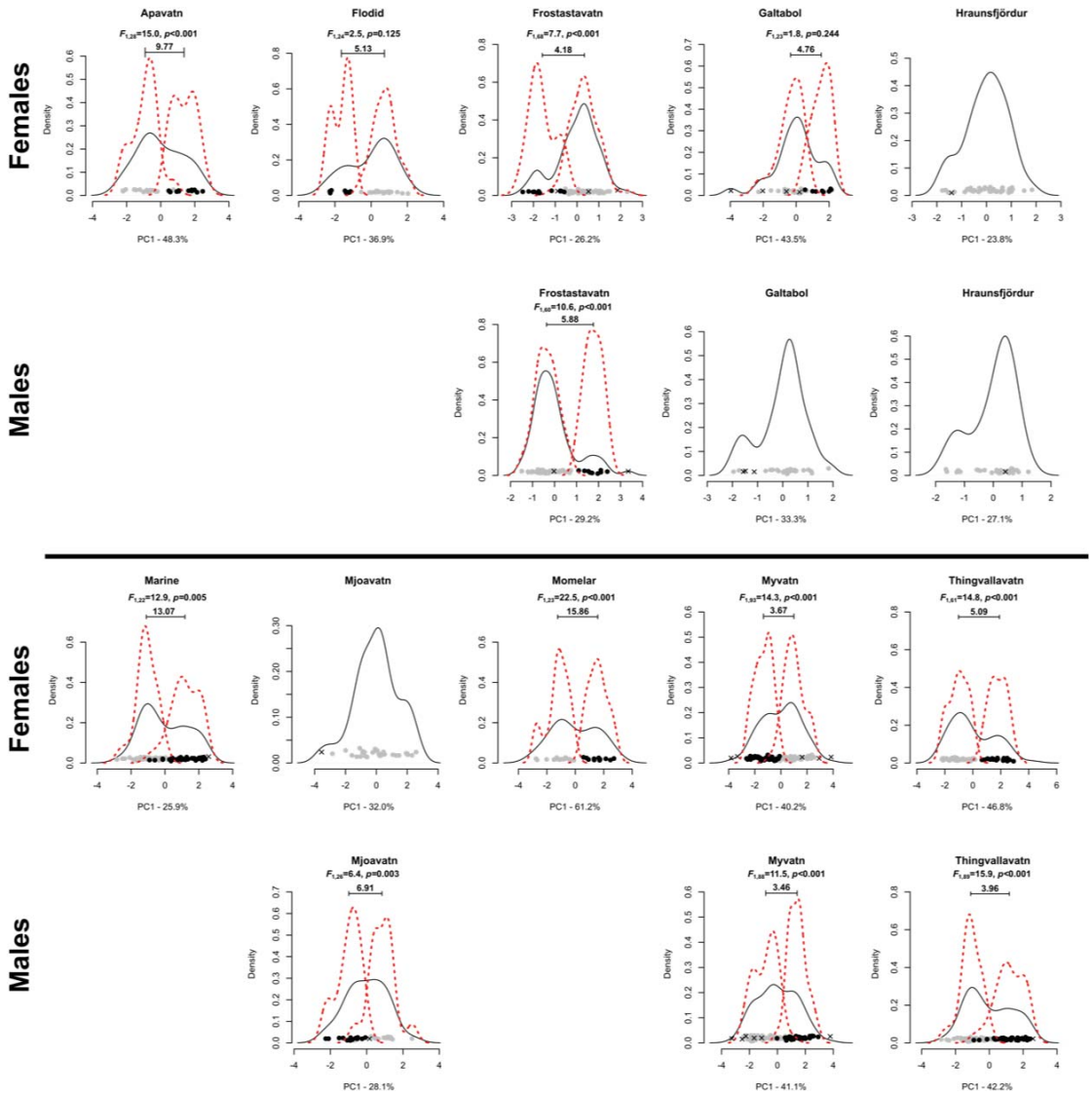

Figure 4: *Kernel density function of the PC1 scores in each lake calculated for females and males separately. Kernel densities are shown for all individuals combined (black line) or separately for each identified multivariate mode (red dashed line). Crosses indicate individuals that were excluded by the clustering algorithm (see text for details). Above the density plots we indicate the p values between modes based on a MANOVA for all traits using clusters as factor as well as the Mahalanobis distances between the identified modes. Empty panels indicate cases where sample size was too small to perform a clustering analysis. Note that*

the PC1 axis only reflects the major axis of multivariate trait variation and may thus slightly differ from the multivariate cluster analysis.

The *post hoc* ANOVA decomposition indicates evidence for parallelism in trait divergence between sympatric phenotype clusters in different lakes (Figure 5, Table S2). Especially differentiation in body shape-related traits, i.e. body depth and the pelvic girdle structure, occurs wherever we found evidence for the existence of two clusters with at least one trait significantly different (i.e. $p < 0.05$; Figure 5, Table S2). Other recurrent axes of divergence exist among head-shape related traits and fin sizes, with at least one trait significantly different in ten out of twelve cases where we find two clusters. Significant differentiation in defense related traits occurred in nine out of twelve cases. Overall, the traits that are divergent in the marine population are mostly unrelated from those that are divergent in freshwater, which may indicate different mechanisms of phenotypic differentiation. The number of statistically differentiated traits and hence the dimensionality of phenotypic differentiation was largest in the two largest lakes (Mývatn: females – 12, males – 11 out of 18 traits; Thingvallavatn: females – 12, males – 16 out of 18 traits). Similarly highly dimensional differentiation was found for females in Mómelar (11 out of 18 traits), whereas less than 10 traits were significantly differentiated between clusters in all other lakes (Figure 5, Table S2). The number of statistically differentiated traits for all cases with a bimodal phenotypic distribution (Figure 5, Table S2) was significantly positively correlated with lake surface ($F_{1,8} = 10.7$, $p = 0.011$; Figure 6), the maximal depth of a lake ($F_{1,8} = 5.5$, $p = 0.047$) and the approximated lake volume (surface multiplied with maximal depth: $F_{1,8} = 5.5$, $p = 0.047$), hence with ecosystem size, but neither with the distance from the sea ($F_{1,8} = 0.2$, $p = 0.651$) nor with elevation ($F_{1,8} = 1.1$, $p = 0.323$), based on linear models using sex as a fixed factor.

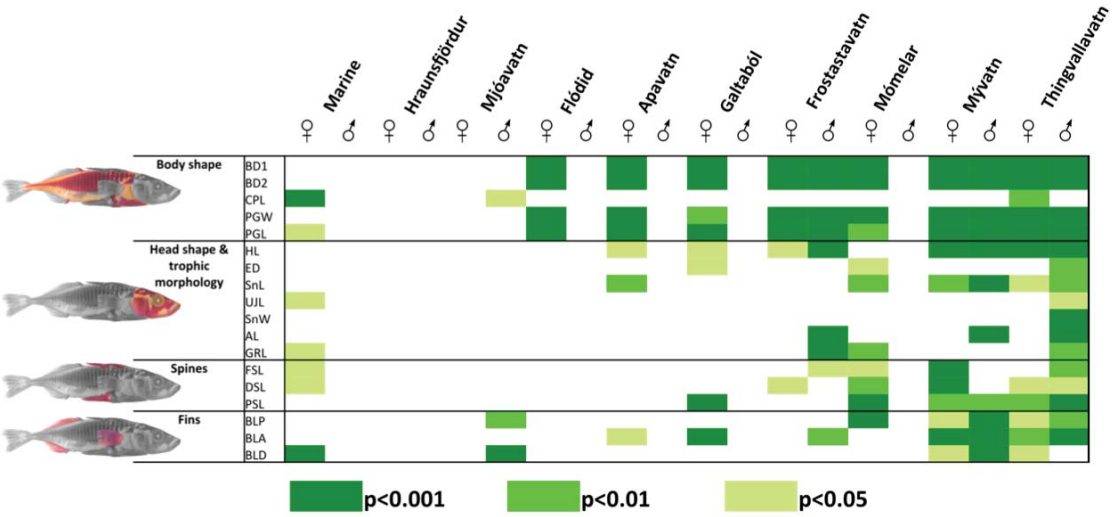

Figure 5: *The ecological speciation continuum in Icelandic lake stickleback. Graphical representation of the phenotypic differentiation between the phenotypic clusters based on a post hoc ANOVA decomposition of each MANOVA performed for each lake and sex where two clusters were identified (see main text for details and*

Table S1 for the actual statistical values). Abbreviations are as follow: BD1 – body depth after the 1st dorsal spine, BD2 – body depth after the 2nd dorsal spine, CPL – caudal peduncle length, PGW – pelvic girdle width, PGL – pelvic girdle length, HL – head length, ED – eye diameter, SnL – snout length, UJL – upper jaw length, SnW – snout width, AL – gill arch length, GRL – length of the second gill raker, FSL – length of the 1st dorsal spine, SSL – length of the 2nd dorsal spine, PSL – length of the pelvic spine, TLP - total length of the pelvic fin, BLA - basal length of the anal fin, BLD - basal length of the dorsal fin.

The multivariate differentiation between clusters within a lake based on Mahalanobis distance (Figure 4) was not significantly associated with lake surface ($F_{1,8}$ = 1.1, p = 0.321), nor with the maximal depth of a lake ($F_{1,8}$ = 0.5, p = 0.501), the distance from the sea ($F_{1,8}$ = 0.1, p = 0.727) or elevation ($F_{1,8}$ = 0.4, p = 0.551), based on linear models using sex as a fixed factor. Mahalanobis distances between clusters was highest for females in Mómelar (Mahalanobis distance: 15.86) and the Marine population (13.07). For Mývatn and Thingvallavatn that showed a high dimensionality of phenotypic differentiation, the Mahalanobis distances between clusters were among the lowest: (Mývatn - males: 3.46, females: 3.67; Thingvallavatn males: 3.96, females: 5.09; Figure 4).

The third step towards ecological speciation: neutral marker differentiation

The STRUCTURE analyses found indication for at least two genetic clusters in Mývatn (Table 2): some individuals showed more than 80% assignment probability to one or the other genetic cluster (Figure S1), but no individual was 100% assigned to either cluster (note that 100% assignments occur when K>3). The pairwise F_{ST} between the individuals that were assigned with ≥75% probability to the less abundant cluster (red in Figure S1) and all other individuals was significantly increased (F_{ST} = 0.151, p < 0.001) above the overall level of genetic differentiation among sampling sites (global F_{ST} = 0.031, p = 0.001). Individuals with high assignment probability to the less abundant genetic cluster were mainly sampled from the two mud sites (sites Mývatn 1 and 2), where the Mývatn 1 site seems almost entirely composed of the globally less common genotype cluster, whereas site 2 appears to have relatively even numbers of individuals belonging to both groups. Our sample from the Myvatn 1 site is very highly significantly genetically differentiated from all other samples from this lake (Table S1). Remarkably, this population is geographically surrounded by populations dominated by the other genotype cluster, and isolation by distance cannot explain its strong genetic differentiation (Mantel test: r =-0.091, p = 0.321).

STRUCTURE did not find anything in the other lakes (Table 2). However, STRUCTURE is known to be constrained by small number of loci when F_{ST} are small (Hubisz *et al.*, 2009). When we used the assignment to phenotypic clusters to make groups, we found the phenotypic groups among the males from Thingvallavatn to be significantly genetically differentiated (F_{ST} = 0.009, p = 0.035), but found no other significant differentiation between groups (Table 2). However, the global F_{IS} values were significant not only in Mývatn and

Thingvallavatn , but also in Mómelar and Hraunsfjördur (Table 2) indicating the possibility of some genetic substructure.

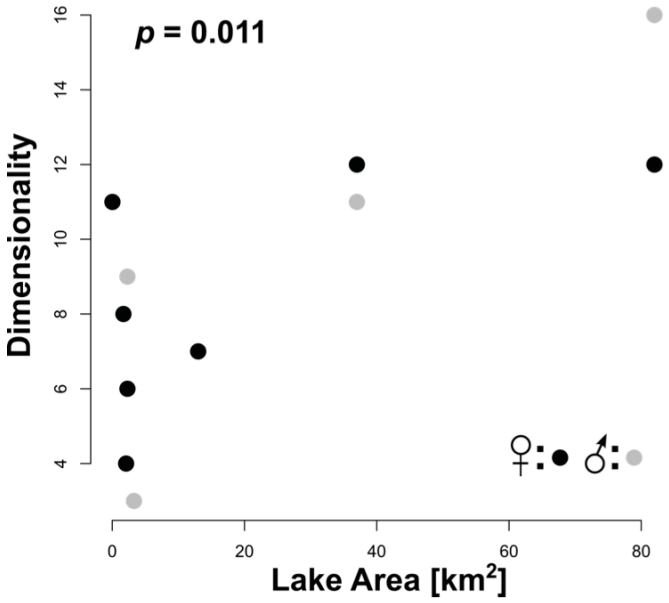

Figure 6: *Relationship between lake area (km2) and the dimensionality of sympatric ecotype formation. Dimensionality is measured as the number of significant differences between identified phenotypic clusters for both females (black) and males (grey; see Figure 5 & Table S2). The p value derives from linear model using sex as a fixed factor (see main text for details).*

In a few systems the phenotypic clusters correlated with body size (SL), age or the substrate type from which they were sampled (Table S2): For Frostastavatn, individuals assigned to the different clusters among females differed significantly in size and were not randomly distributed in terms of substrates (mud versus lava). Individuals in the more abundant cluster derive mainly from the lava substrate and were significantly smaller than individuals from the less abundant cluster. Individuals from the two phenotypic male modes also tended to be non-randomly distributed over substrate types (p = 0.083). Males assigned to the different clusters differed in age (p = 0.042), where individuals in the less common cluster were on average 2.1 years old as opposed to 1.8 years in the less common cluster. The statistical models for substrate and age fitted the phenotypic data equally well (ΔAIC: 1.19). Substrate and sampling site were significantly different between the phenotypic clusters in Mývatn, and this was true for both sexes (Table S2), with sampling site better supported (females: ΔAIC: 22.64, males: ΔAIC: 16.71). The phenotypic clusters among males in Thingvallavatn were also non-randomly distributed over substrate types (p = 0.029), and a strong trend was also seen in females (p = 0.062). Only age was different between the phenotypic clusters among females in the marine population (p = 0.020).

Table 2: *Summary table for population genetic indices calculated for each lake and the marine population. Indicated are the sample size (N) of genotyped individuals per sex and the total number of available individuals being genotyped for nine microsatellites (note: for some individuals, sex could not be determined). K indicates the number of genetic clusters identified by* STRUCTURE *for cases where > 20 individuals were available. Additionally, the global inbreeding coefficient (F_{IS}) for all individuals within each studied system and separately for each sex, the global F_{IS} as well as the pairwise F_{ST} between the identified sex specific phenotypic modes within a lake are given (see main text for details).*

	Sample size			STRUCTURE			Global F_{IS}			F_{ST} among modes	
Site	$N_{females}$	N_{males}	N_{all}	$K_{females}$	K_{males}	K_{all}	**Females**	**Males**	**All**	**Females**	**Males**
Apavatn	30	11	41	1	-	1	0.039	0.012	0.031	0.008	-
Flódid	26	4	30	1	-	1	0.014	0.000	0.008	0.001	-
Frostastavatn	73	58	131	1	1	1	-0.067	-0.064	-0.065	-0.015	0.018
Galtaból	27	26	53	1	1	1	-0.088	0.104°	0.009	-0.005	-
Hraunsfjördur	42	30	72	1	1	1	0.074**	0.013	0.048*	-	-
Mjóavatn	17	25	42	-	1	1	0.025	0.030	0.026	-	-0.009
Mómelar	25	9	34	1	-	1	0.011	0.132°	0.070*	-0.015	-
Mývatn	93	95	195	1	1(2)†	1(2)†	0.045*	0.103***	0.077***	0.002	0.006
Thingvallavatn	51	83	148	1	1	1	0.040°	0.035°	0.043**	0.000	0.009*
Marine	31	14	45	1	-	1	0.029	0.022	0.024	0.012	-

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ° $0.05 < p < 0.10$

† Although no clear clustering was achieved, the STRUCTURE runs indicate a potential substructure into two clusters (see Figure S1 and main text for details).

Discussion

Stages in the sympatric speciation continuum

The very early stages in adaptive radiation and ecological speciation, after colonization of a new adaptive zone, may often be characterized by an expansion of phenotypic variation (Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999; Yoder *et al.*, 2010). This would be followed by a transition from unimodal phenotypic variation to differentiated phenotypic clusters (Doebeli & Dieckmann, 2000; Leimar *et al.*, 2008; Hendry, 2009; Seehausen, 2009; Nosil, 2012). And finally, gene flow would become increasingly suppressed as these groups become increasingly reproductively isolated (Seehausen et al., 2008a). Studying freshwater lake populations of the threespine stickleback in Iceland that range in age from 50 years to several thousand years, we find support for these predictions. Phenotypic variation in all but one lake significantly exceeded that in a marine population that can be considered representative of the ancestral condition. The one exception is Hraunsfjödur, a 50 years young isolate from the Sea. Colonization of freshwater lakes from the Sea was thus generally associated with a morphospace expansion. We found phenotypic variation was unimodal in some lakes, including the youngest population (Hraunsfjödur, 50 years), but it ranged from weakly to strongly bimodal in other, older populations. The phenotypically defined groups were associated with different substrate types in three lakes and in two of these also with sampling site and in one of them with fish age. Finally, we found evidence for neutral marker differentiation among phenotypic clusters only in the two largest lakes, suggesting an advanced stage of ecological speciation (Hendry, 2009; Seehausen, 2009; Nosil, 2012; Feder *et al.*, 2012). Although variation in lake size and lake depth did not significantly explain the observed phenotypic variation, the dimensionality of phenotypic differentiation between ecotypes was significantly positively correlated with lake surface (Figure 6) and lake depth and hence ecosystem size.

Gene flow and the potential for diversification

The role of gene flow in either constraining or facilitating adaptive population diversification is a long-standing debate (see Räsänen & Hendry, 2008 for a review). On the one hand, gene flow from outside a population may impede adaptive differentiation and speciation by diluting locally adapted alleles, homogenizing gene pools and thus preventing the formation of co-adapted gene complexes (Kawecki & Ebert, 2004; Räsänen & Hendry, 2008; Nosil & Feder, 2012). On the other hand, gene flow may increase adaptive variation and heritability, and this may be especially important in isolated populations (Seehausen *et al.*, 2008b; Abbott *et al.*, 2013). Intra- or interspecific hybridization may even facilitate the evolution of new species through ecological speciation and entire adaptive radiations (Seehausen, 2004; Nolte & Tautz, 2009; Abbott *et al.*, 2013).

Consistent with a pattern of increasing isolation, we find that the degree of genetic differentiation (F_{ST}) of lake populations from the marine population increases with elevation of lakes above sea level (Figure 2). The populations in many low lying lakes are also not strongly separated from the marine population in our population tree (Figure 3), whereas all populations from upland lakes sit at the tips of long branches. Three of these upland lakes cluster together despite being geographically very distinct. This pattern may reflect different colonization waves by sticklebacks to Icelandic freshwater lakes, where depending on the distinct glaciation history during the last Ice Age, some upland lakes became available for colonization earlier than others (Le Breton *et al.*, 2010). Overall, our findings suggest that recent or past gene flow from the Sea is negatively correlated with elevation of lakes above Sea level. Concomitantly, the level of heterozygosity and thus standing genetic variation in lakes decreases with elevation too. Putting all these observations together, the potential for adaptive radiation in lake populations of Icelandic stickleback might be predicted to be highest at intermediate elevations, where current gene flow from the Sea is absent or very weak, but standing genetic variation is still moderately high (Kawecki & Ebert, 2004; Räsänen & Hendry, 2008).

Intralacustrine diversification: Ecological opportunity and dimensionality

Theory predicts a positive correlation between the number of species that can evolve during an adaptive radiation and ecosystem size (Simpson, 1953; Schluter, 2000; Gavrilets & Vose, 2005), which is commonly approximated by area or habitat size (Gavrilets & Losos, 2009). Significant relationships between habitat size and the number of species that emerged through an adaptive radiation have been found in a few cases, most notably *Anoles* lizards (Losos & Schluter, 2000), Galapagos land snails (Parent & Crespi, 2006)), and African cichlid fish (Wagner *et al. submitted*). Yet, habitat size does not solely explain the occurrence or the extent of some freshwater fish radiations (Vamosi, 2003; Ormond *et al.*, 2011; Wagner *et al.*, 2012, Wagner *et al. submitted)*. Especially adaptive radiations of freshwater fish in postglacial lakes seem to be rather related to environmental factors (Seehausen *et al.*, 1997; Vonlanthen *et al.*, 2012), including oxygen depletion (Landry *et al.*, 2007), differences in available prey size (Landry & Bernatchez, 2010) or in the case of stickleback radiating in extremely species-depauperate systems, interspecific interactions with the only other fish occurring in these systems (Vamosi, 2003; Ormond *et al.*, 2011).

We find neither the phenotypic variation within a lake nor the occurrence of intralacustrine diversification to be associated with lake size or depth. This is consistent with similar studies on Canadian threespine stickleback (Vamosi, 2003; Ormond *et al.*, 2011). The dimensionality of ecotypic differentiation, defined as the number of differentiated traits between phenotypic clusters, is however significantly related to both lake size and the maximal depth of a lake. Mómelar, being the smallest of our studied lakes is in this regard exceptional as the level of dimensionality of ecotypic differentiation is only slightly less than in the two largest lakes. This population is genetically quite distinct and may be of considerable age (Figure 3, Table S1). In addition, it is the only lake that we

studied where sticklebacks occur in the absence of any other fish species (Lucek *personal observation*). Both age and ecological release from interspecific competition may have facilitated the evolution of phenotypic diversity and ecotypic differentiation (Bolnick *et al.*, 2010). Overall, all upland lakes above 100m a. s. l., except Mjóavatn tend to have a higher dimensionality than lowland lakes (Figure 5). This may indicate that time for evolution is important too in addition to ecosystem size.

We find in some lakes, including the two largest – Mývatn and Thingvallavatn, that the phenotype clusters are significantly associated with substrate type (Table S2). This is consistent with prior studies on these lakes (Kristjánsson et al., 2002a; Ólafsdóttir et al., 2007a; Millet *et al.*, 2013) and indicates that habitat heterogeneity may have promoted ecological speciation in Icelandic stickleback. However, it is possible that spatial heterogeneity that is not related to substrate could play a role in Mývatn and Thingvallavatn too because in both cases we find that sampling site itself also explains variation in the individual assignment to phenotypic modes. These effects of spatial and habitat heterogeneity may be driving the relationship between ecosystem size and phenotypic dimensionality of differentiation. This is in line with theoretical predictions for intraspecific diversification in heterogeneous habitats, leading to the evolution of multiple phenotypic modes (Doebeli & Dieckmann, 2003; Leimar *et al.*, 2008; Débarre, 2012).

Parallelisms in the intralacustrine evolution of ecotypes

The traits that are associated with sympatric ecotype formation in Icelandic stickleback show parallel divergence trends among replicate lakes. Ecotypic differentiation in seven of nine lakes involves body depth and pelvic girdle dimensions (Figure 5, Table S2). Differences in body depth has been found among distinct stickleback ecotypes in many other systems (Reid & Peichel, 2010; Voje *et al.*, 2013; Lucek *et al.*, 2013; Ravinet *et al.*, 2013b). These differences are thought to be of adaptive relevance, where plankton feeding fish are generally more streamlined than benthic feeding fish, facilitating both foraging and cruising in open water (Reid & Peichel, 2010). The ecotypic differentiation that we observed in body depth and to a lesser extent in head shape and gill raker length are consistent with adaptation to different trophic resources (Schluter & McPhail, 1992; Walker, 1997). Differences in feeding strategies between Icelandic stickleback collected from different substrates has previously been found (Kristjánsson *et al.*, 2002a) and may thus importantly contribute to the evolution of distinct sympatric phenotypes in Icelandic lake stickleback.

Differences in fin size and anti-predator defense trait are also recurrent, especially for the two largest lakes (Figure 5). These differences are also thought to be adaptive, where differences in fin sizes relate to different sustained swimming capabilities (Reid & Peichel, 2010). Differences in anti-predator defense trait thought to be associated with variation in predation regimes, where gape-limited predators such as birds and fish select for increased spine lengths (Reimchen, 1994) and grappling predators like insect larvae select for reduced

armor (Reimchen, 1980; 1994). However, large predatory insect larvae seem to be rare in Iceland and other selective agents may underlie the observed differentiation in spine lengths (Doucette *et al.*, 2004; Lucek *et al.*, 2012b).

In two instances – the marine population and Frostastavatn, the identified phenotypic clusters represent different age classes (Table S2). In the latter case, both sexes form distinct age-related phenotypic clusters that differ in body shape and to a lesser extent in defense and head morphology. The individuals assigned to each cluster were furthermore statistically associated with substrate. Hence, foraging behavior may differ among age classes in these populations. This can itself be adaptive (Dill, 1983), where the observed phenotypic differentiation would facilitate resource partitioning among age classes. Alternatively though, there could be two ecotypes that differ in longevity. Longevity is known to be a life history traits that has diverged between stickleback ecotypes in other systems (Baker *et al.*, 2005; Moser *et al.*, 2012; Lucek *et al.*, 2012a). Future research into these systems should address this.

Genetic differentiation and reproductive isolation

Neutral genetic structure within lakes was weak (Table 2). The program STRUCTURE identified evidence for genetic structure only in Mývatn (Figure S1). However, the power of STRUCTURE in inferring genetic clusters from a limited number of markers is constrained when genetic differentiation is weak (Hubisz *et al.*, 2009). For instance, it typically fails to find genetic structure among closely related sympatric species such as cichlid fish in Lake Victoria (Magalhaes *et al.*, 2009; 2010), but the same program finds the same populations of the same species highly significantly structured when fed with thousands of markers (I. Keller *et al.*, 2013). Two additional lines of evidence indicate further genetic population structure among our stickleback samples. First, we find some branches within the population trees for lakes Mývatn and Thingvallavatn with significant bootstrap support. Second, the coexistence of groups of nonrandomly mating individuals (Wahlund effect), is indicated in four lakes that show a significant global inbreeding coefficients (F_{IS}) (Bernatchez & C. Wilson, 1998). Third, pairwise F_{ST} values suggest significant genetic differentiation between distinct sampling sites in lakes Hraunsfjördur, Thingvallavatn and Mývatn (Table S1). In the latter case, individuals from a single site (Mývatn 1) differ genetically from all other sites, which is congruent with the STRUCTURE analysis (Figure S1). In contrast, significant genetic differentiation (F_{ST}) between phenotypic clusters occurs only in Thingvallavatn (Table 2). The overall weak genetic structure among phenotypic clusters contrasts with some previous studies that found genetic differentiation between populations inhabiting distinct substrates in Hraunsfjördur (Ólafsdóttir et al., 2007b), Mývatn (Ólafsdóttir et al., 2007c) and Thingvallavatn (Ólafsdóttir & Snorrason, 2009). In the latter case, it is possible that the use of many phenotype-linked markers in their study (Ólafsdóttir & Snorrason, 2009), but few in ours, could explain this difference in the extent of genetic divergence between ecologically defined clusters. Genetic differentiation in mainly phenotype-linked markers would suggest a very early stage in the

speciation continuum, where divergent selection acts on small regions in the genome (Hendry, 2009; Nosil, 2012; Feder *et al.*, 2012).

Conclusions

We find that colonization of lakes from the Sea is generally associated with an increase in intrapopulation phenotypic variation in Icelandic stickleback, which we consider evidence for the first stage in ecological speciation. Next, we find that sympatric diversification into phenotypically distinct ecotypes within lakes is a recurrent phenomenon among these populations. We suggest this marks the second stage in the speciation continuum. Both ecosystem size and the time since colonization seem to predict the dimensionality of sympatric ecotype formation and the occurrence of neutral genetic differentiation. We suggest this signals is the third stage, where gene flow between divergent groups is sufficiently constrained to detect differentiation in neutral markers. We find evidence for this in the two largest lakes, suggesting that ecosystem size predicts how far speciation may proceed.

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Table S1: *Pairwise genetic differentiation (F_{ST}) among all sampled sites (lower triangle) with the respective p values based on 1000 bootstrap replicates. Significant ($p < 0.05$) F_{ST} values are highlighted in bold.*

	Marine	Hraunsfördur 1	Hraunsfjördur 2	Apavatn	Mómelar	Flodid	Galtaból	Miójavatn	Frostastavatn 1	Frostastavatn 2	Mývatn 1	Mývatn 2	Mývatn 3	Mývatn 4	Mývatn 5	Mývatn 6	Thingvallavatn 1	Thingvallavatn 2	Thingvallavatn 3	Thingvallavatn 4	Thingvallavatn 5
Marine	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Hraunsfördur 1	**0.029**	-	0.041	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Hraunsfjördur 2	**0.044**	**0.007**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Apavatn	**0.027**	**0.032**	**0.024**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Mómelar	**0.157**	**0.176**	**0.188**	**0.190**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Flódid	**0.021**	**0.037**	**0.049**	**0.034**	**0.192**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Galtaból	**0.371**	**0.391**	**0.420**	**0.400**	**0.551**	**0.331**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Miójavatn	**0.306**	**0.289**	**0.322**	**0.317**	**0.464**	**0.248**	**0.146**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Frostastavatn 1	**0.343**	**0.344**	**0.362**	**0.350**	**0.541**	**0.317**	**0.556**	**0.523**	-	0.191	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Frostastavatn 2	**0.352**	**0.357**	**0.372**	**0.358**	**0.551**	**0.326**	**0.546**	**0.523**	0.003	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Mývatn 1	**0.298**	**0.301**	**0.318**	**0.289**	**0.476**	**0.306**	**0.653**	**0.590**	**0.565**	**0.572**	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Mývatn 2	**0.263**	**0.267**	**0.278**	**0.244**	**0.461**	**0.260**	**0.637**	**0.571**	**0.545**	**0.548**	**0.066**	-	0.460	0.522	0.259	0.192	0.001	0.001	0.001	0.001	0.001
Mývatn 3	**0.267**	**0.275**	**0.290**	**0.250**	**0.467**	**0.266**	**0.648**	**0.583**	**0.554**	**0.554**	**0.119**	0.000	-	0.594	0.342	0.430	0.001	0.001	0.001	0.001	0.001
Mývatn 4	**0.284**	**0.291**	**0.302**	**0.266**	**0.482**	**0.282**	**0.640**	**0.585**	**0.548**	**0.548**	**0.103**	-0.002	-0.003	-	0.263	0.863	0.001	0.001	0.001	0.001	0.001
Mývatn 5	**0.281**	**0.285**	**0.296**	**0.261**	**0.477**	**0.279**	**0.638**	**0.576**	**0.550**	**0.550**	**0.104**	0.004	0.002	0.003	-	0.251	0.001	0.001	0.001	0.001	0.001
Mývatn 6	**0.260**	**0.269**	**0.279**	**0.241**	**0.460**	**0.256**	**0.624**	**0.562**	**0.535**	**0.536**	**0.104**	0.006	0.000	-0.006	0.003	-	0.001	0.001	0.001	0.001	0.001
Thingvallavatn 1	**0.172**	**0.164**	**0.174**	**0.178**	**0.335**	**0.213**	**0.514**	**0.429**	**0.548**	**0.551**	**0.485**	**0.455**	**0.465**	**0.470**	**0.466**	**0.444**	-	0.565	0.511	0.939	0.508
Thingvallavatn 2	**0.192**	**0.175**	**0.182**	**0.194**	**0.352**	**0.230**	**0.516**	**0.428**	**0.564**	**0.567**	**0.495**	**0.467**	**0.478**	**0.481**	**0.476**	**0.453**	-0.002	-	0.069	0.357	0.289
Thingvallavatn 3	**0.160**	**0.172**	**0.182**	**0.172**	**0.326**	**0.202**	**0.537**	**0.439**	**0.565**	**0.567**	**0.502**	**0.469**	**0.480**	**0.488**	**0.482**	**0.457**	-0.002	0.015	-	0.203	0.015
Thingvallavatn 4	**0.172**	**0.163**	**0.176**	**0.180**	**0.333**	**0.212**	**0.483**	**0.408**	**0.523**	**0.528**	**0.461**	**0.435**	**0.443**	**0.448**	**0.445**	**0.424**	-0.006	0.001	0.005	-	0.269
Thingvallavatn 5	**0.177**	**0.157**	**0.159**	**0.176**	**0.337**	**0.215**	**0.559**	**0.449**	**0.582**	**0.584**	**0.503**	**0.470**	**0.482**	**0.489**	**0.481**	**0.461**	-0.001	0.005	**0.026**	0.003	-

Table S2: *Number of identified phenotypic modes and MANOVA results for phenotypic traits using modes as factors, calculated for each lake and for each sex separately. P values for traits are based on a post hoc ANOVA decomposition of each MANOVA analysis. Significant p values are highlighted in bold, p values with 0.05 < p 0.10 are highlighted in italics. See main text for abbreviations.*

Females

	Apavatn	**Flódid**	**Frostastavatn**	**Galtaból**	**Hraunsfjördur**	**Marine**	**Mjóavatn**	**Mómelar**	**Mývatn**	**Thingvallavatn**
Sample size	30	26	70	25	42	24	25	25	95	63
# phenotypic modes	2	2	2	2	1	2	1	2	2	2
MANOVA	$F_{1,28}$ = 15.0	$F_{1,24}$ = 2.5	$F_{1,68}$ = 7.7	$F_{1,23}$ = 1.8	-	$F_{1,22}$ = 12.9	-	$F_{1,23}$ = 22.5	$F_{1,93}$ = 14.3	$F_{1,61}$ = 14.8
p	**<0.001**	0.125	**<0.001**	0.244	-	**0.005**	-	**<0.001**	**<0.001**	**<0.001**
Body shape										
Body depth 1	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	0.772	-	**<0.001**	**<0.001**	**<0.001**
Body depth 2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	0.849	-	**<0.001**	**<0.001**	**<0.001**
Caudal peduncle length	0.201	0.965	*0.081*	0.580	-	**<0.001**	-	*0.051*	0.680	**0.002**
Pelvic girdle width	**<0.001**	**<0.001**	**<0.001**	**0.005**	-	*0.083*	-	**<0.001**	**<0.001**	**<0.001**
Pelvic girdle length	**<0.001**	**0.001**	**<0.001**	**<0.001**	-	**0.022**	-	**0.004**	**<0.001**	**<0.001**
Spines										
Length 1st dorsal spine	0.874	0.744	0.189	0.114	-	**0.021**	-	**0.014**	**<0.001**	0.237
Length 2nd dorsal spine	0.518	0.911	**0.012**	*0.081*	-	**0.010**	-	**0.003**	**<0.001**	**0.015**
Pelvic spine length	0.727	0.578	0.691	**<0.001**	-	0.236	-	**<0.001**	**0.007**	**0.001**
Head shape & trophic morphology										
Snout length	**0.002**	0.531	0.406	0.332	-	0.320	-	**0.005**	**0.002**	**0.011**
Upper jaw length	0.186	*0.078*	0.825	0.244	-	**0.027**	-	*0.053*	*0.091*	0.106
Snout width	0.246	0.296	0.279	0.911	-	0.533	-	0.400	*0.063*	0.199
Length 2nd gill raker	0.939	0.328	0.129	0.261	-	**0.012**	-	**0.002**	*0.084*	0.371
Gill arch length	0.394	0.817	0.419	0.392	-	0.286	-	0.280	0.185	0.356
Head length	**0.015**	0.144	**0.013**	**0.026**	-	0.601	-	*0.080*	**<0.001**	**<0.001**
Eye diameter	0.791	0.410	0.591	**0.042**	-	0.267	-	**0.013**	0.711	0.224
Fins										
Total length pelvic fin	0.185	0.746	0.473	0.680	-	0.129	-	**<0.001**	**0.042**	**0.034**
Basal length anal fin	**0.013**	0.303	0.980	**0.009**	-	**0.023**	-	0.215	**<0.001**	**0.004**
Basal length dorsal fin	0.200	0.747	*0.079*	0.104	-	**<0.001**	-	0.514	**0.045**	**0.022**

Males

	Apavatn	Flódid	Frostastavatn	Galtaból	Hraunsfjördur	Marine	Mjóavatn	Mómelar	Mývatn	Thingvallavatn
Sample size	11	4	58	26	30	14	25	9	90	91
# Modes	-	-	2	1	1	-	2	-	2	2
MANOVA	-	-	$F_{1,60} = 10.6$	-	-	-	$F_{1,23} = 6.4$	-	$F_{1,88} = 11.5$	$F_{1,89} = 15.9$
p	-	-	**<0.001**	-	-	-	**0.003**	-	**<0.001**	**<0.001**
Body shape										
Body depth 1	-	-	**<0.001**	-	-	-	0.299	-	**<0.001**	**<0.001**
Body depth 2	-	-	**<0.001**	-	-	-	0.152	-	**<0.001**	**<0.001**
Caudal peduncle depth	-	-	0.403	-	-	-	**0.028**	-	0.916	0.346
Pelvic girdle width	-	-	**<0.001**	-	-	-	0.953	-	**<0.001**	**<0.001**
Pelvic girdle length	-	-	**<0.001**	-	-	-	0.118	-	**<0.001**	**<0.001**
Spines										
Length 1st dorsal spine	-	-	**0.012**	-	-	-	0.104	-	0.181	**0.008**
Length 2nd dorsal spine	-	-	0.169	-	-	-	*0.054*	-	0.478	**0.018**
Pelvic spine length	-	-	0.222	-	-	-	*0.058*	-	**0.013**	**<0.001**
Head shape & trophic morphology										
Snout length	-	-	0.533	-	-	-	0.643	-	**<0.001**	**0.004**
Upper jaw length	-	-	0.763	-	-	-	*0.080*	-	0.284	**0.031**
Snout width	-	-	0.499	-	-	-	0.371	-	0.587	**<0.001**
Length 2nd gill raker	-	-	**0.001**	-	-	-	0.277	-	0.178	**0.010**
Gill arch length	-	-	**0.001**	-	-	-	0.424	-	**<0.001**	**<0.001**
Head length	-	-	**0.004**	-	-	-	0.649	-	**<0.001**	**<0.001**
Eye diameter	-	-	0.516	-	-	-	0.552	-	0.228	**0.013**
Fins										
Total length pelvic fin	-	-	*0.080*	-	-	-	**0.002**	-	**<0.001**	**0.007**
Basal length anal fin	-	-	**0.007**	-	-	-	0.968	-	**<0.001**	**<0.001**
Basal length dorsal fin	-	-	*0.085*	-	-	-	**<0.001**	-	**<0.001**	0.127

Table S3: *ANOVA summary, testing for an association of the identified phenotypic modes with: fish standard length (SL), fish age, substrate or sampling site. Given are the respective degrees of freedom (d.f.), F values and the corresponding p and AIC values for each location and sex. Results are only shown for models with p < 0.1. Cases where 0.05 < p < 0.1 are highlighted in italics.*

Location	**Sex**	**Response variable**	**d.f.**	***F***	***p***	**AIC**
Frostastavatn	Females	SL	1,68	4.8	0.032	67.63
		Substrate*	1,68	4.5	0.038	67.96
	Males	Age	1,60	4.3	0.042	48.26
		*Substrate**	*1,60*	*3.1*	*0.083*	*49.45*
Marine	Females	Age	1,22	6.3	0.020	34.63
Mývatn	Females	Substrate	2,92	5.2	0.007	133.99
		Sampling site	5,89	9.0	<0.001	111.35
	Males	Substrate	2,87	4.4	0.016	125.46
		Sampling site	5,84	7.0	<0.001	108.75
Thingvallavatn	Females	*Sampling site*	*4,58*	*2.4*	*0.062*	*87.86*
	Males	Sampling site	4,86	2.9	0.029	132.22

* Indicates cases where ecotypes and sampling sites are the same

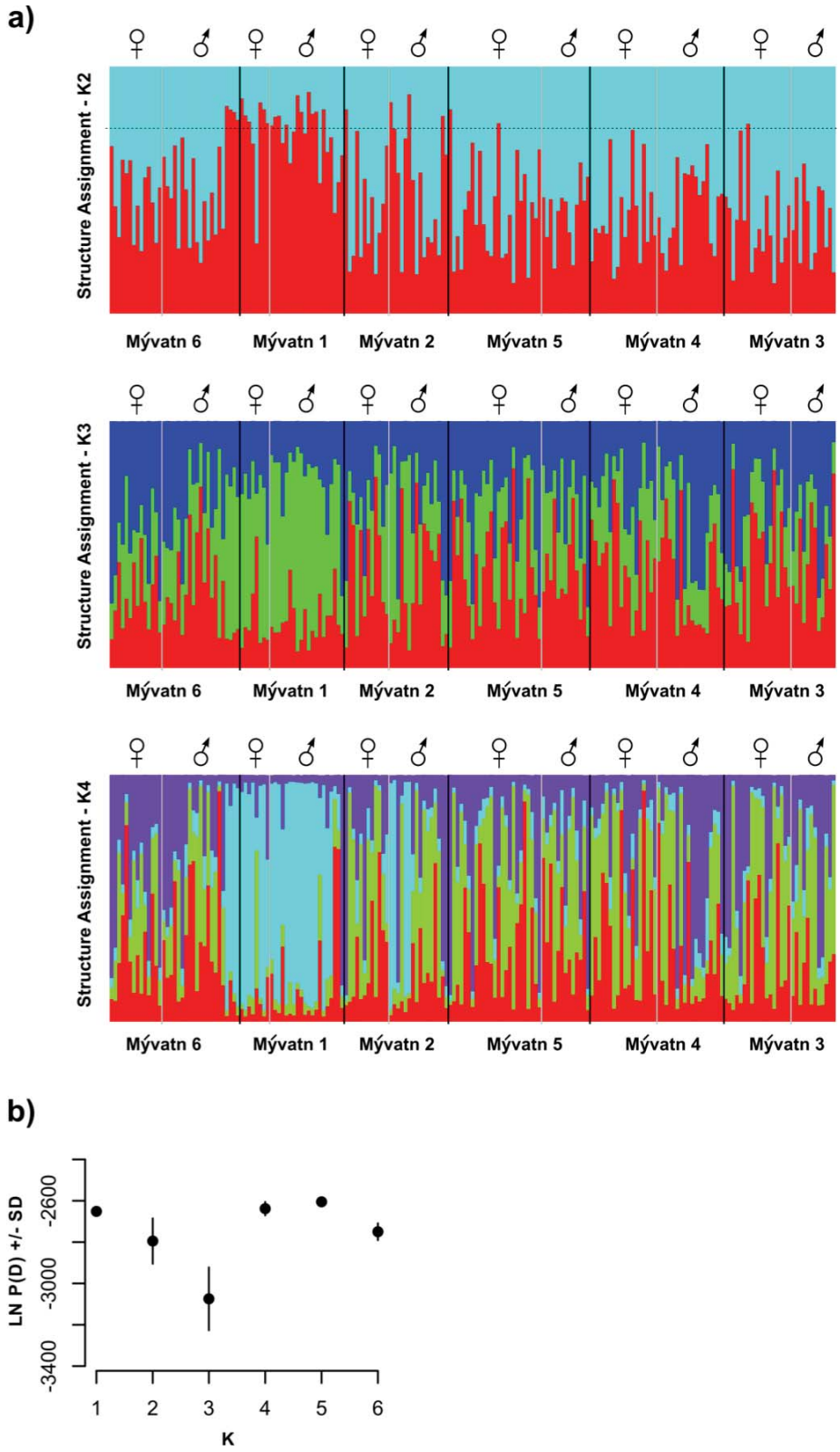

Figure S1: *Structure analysis for Mývatn combining all sites and sexes. a) individual assignment assuming K=2-4. Shown is the best run obtained out of 10 replicates. The dashed line indicates the individual assignment probability > 75% for the red cluster in K=2. Black vertical bars separate sample sites whereas grey vertical bars separate sexes. Site IDs follow Table 1 and are ordered from the northernmost to the southernmost population (see Figure 1). b) log likelihood values for K 1-6 (± 1 SD).*

Supplementary methods

Genomic DNA was extracted from fin tissue sample using a 10% Chelex solution, following the manufacturers protocol (Biorad, California, USA). 10 microsatellites were amplified in a single multiplexing set (Table S2).

The polymerase chain reaction (PCR) reactions consisted of 5 µl Qiagen Multiplexing Solution (Qiagen, Switzerland), 0.95 µl primer mix (Table S4), 3.05 µl dH_2O and 1 µl DNA per reaction. The PCR started with 15 min at 95°C followed by 26 cycles with 95°C for 30 seconds, 53°C for 90 seconds and 72°C for 60 seconds with a final elongation at 60°C for 30 minutes. PCR products were 1:10 diluted and visualized on a ABI 3130XL (Applied Biosystems, USA) following the manufacturers instruction. Alleles were scored using GENEMAPPER v4.0 (Applied Biosystems, USA).

Table S4: *Microsatellites used in this study with their fluorescent and concentration used. Primers and the position in the genome, i.e. linkage group, were obtained from Raeymaekers et al., 2007.*

Marker	QTL	Fluorescent	µl per reaction [10 µM]
Gaest66		Blue	0.1
STN30		Blue	0.1
STN96	2nd spine length	Blue	0.2
STN173		Green	0.05
STN196		Green	0.1
STN130	2nd spine length	Black	0.05
STN174		Black	0.1
STN185		Red	0.1
STN26	1st spine length	Red	0.05

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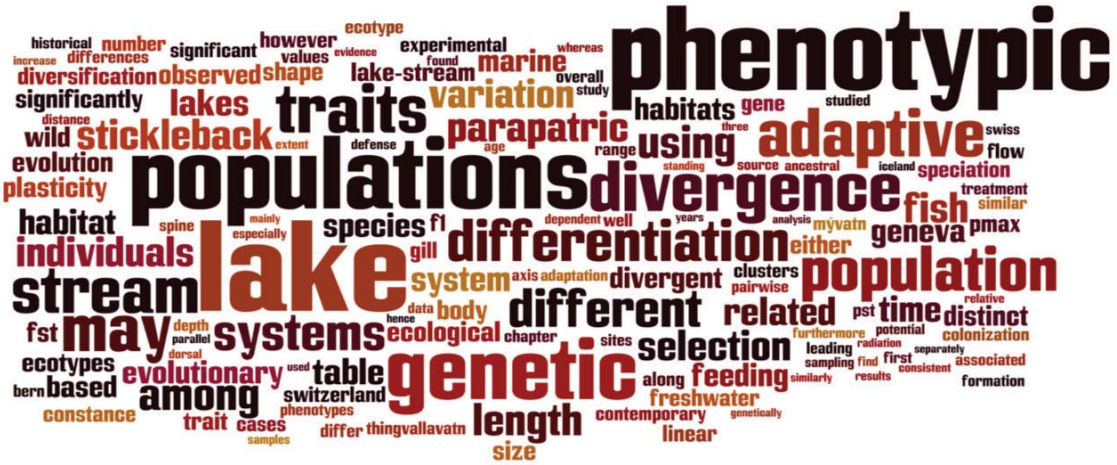

"Summary of this thesis"

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Dear reader, I would like to thank you for your interest reading my thesis up to this point. It summarizes 4.5 years of my life and is based on another 1.5 years that I worked on this topic during my Master Thesis. Yet the summary of it fits on only half a page (see the preceding page) in only a few words. Of course there is much more behind this thesis that lies hidden between its lines and that can hardly be captured with words. This thesis was written in twelve different countries on two continents while being on an airplane, a train, a car, a bus, a couch, or simply in an office. Although it may not seem to be so, you find all my dedication in this piece of work for which I worked days and nights, holidays and weekends over the last years.

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Vi veri veniversum vivus vici

E r k l ä r u n

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S ˜ iengang: PhD in Ecology and Evolution

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&˝e°der Arbei ^ From invasion to diversification - studying the speciation continuum in stickleback

LeiterIn der Arbei ^ Prof. Dr. Ole Seehausen

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Ort/%atum

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Selz O, **Lucek K**, Young K, Seehausen O (2013). Relaxed trait covariance in interspecific cichlid hybrids predicts morphological diversity in adaptive radiations. *Journal of Evolutionary Biology*, in press.

Lucek K, Lemoine M (2012). Introduced guppies being the first record of freshwater fish on the Cape Verdean archipelago. *African Zoology*, 47, 341-344.

Lucek K, Sivasundar A, Seehausen O (2012). Evidence for adaptive diversification during biological invasion. *PLOS ONE,* 7, e49377, 1-6.

Lucek K, Haesler MP, Sivasundar A (2012). When phenotypes do not match genotypes – unexpected phenotypic diversity and potential environmental constraints in Icelandic Stickleback. *Journal of Heredity,* 103, 579-584.

Zeller M, **Lucek K**, Haesler MP, Seehausen O, Sivasundar A (2012). Signals of predation-induced directional and disruptive selection in the threespine stickleback. *Evolutionary Ecology Research*, 14, 193-205.

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