THE ORIGIN OF ALTERNATIVE PHENOTYPES IN FISHES

BY

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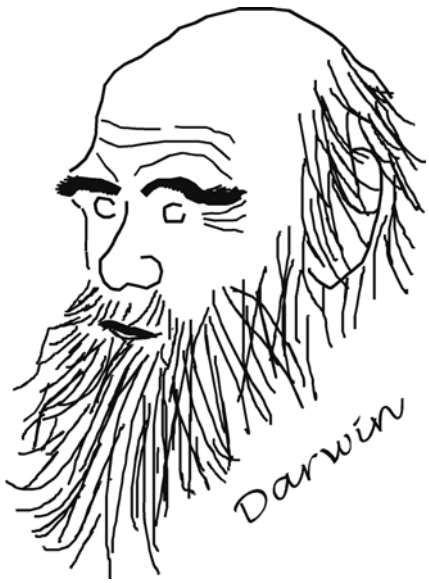
ABSTRACT

A basic aim of evolutionary biology is to explain the enormous diversity among animal and plant species. But also within species there is often large genetic and phenotypic variation, and such variation is necessary for evolution to create new reproductively isolated species. The present thesis is directed to explain differentiation within populations highlighting and discussing the significance of phenotypic plasticity as an evolutionary process that leads to the expression of alternative phenotypes within a species. Such phenotypic expressions are particularly interesting, because the process by which new species are formed typically involves a temporary stage within the splitting species, that is, different heritable and distinct types that coexist within the same population. Such phenotypes may be raw material for full species formation, and the study of alternative-phenotype species should therefore be particularly worthwhile in speciation research. When alternative phenotypes are not entirely genetic they may arise as a result of developmental plasticity, when organisms develop in accordance with local abiotic and biotic conditions. Subject to developmental plasticity, alternative phenotypes, take different developmental routes depending on the local selection pressures, or depending on the environmental conditions experienced during development. Here, laboratory experiments showed that three-spined sticklebacks exhibit alternative phenotypes as a plastic response to physical environment and diet, demonstrating and supporting the idea that environmental inputs modulate the expression of traits through phenotypic plasticity during ontogeny. When, morphological differences arise, discrete morphological characteristics are originated and may be reinforced by the continuous presence of same environmental conditions. Here is demonstrated that these discrete morphological characteristics lead the individuals to specialise on specific prey or habitat types. Moreover, it is showed that plasticity may also play a role in the final stages of species formation, when reproductive isolation completes the speciation process. It is shown that diet-induced morphology has an important influence in mate preferences representing a strong potential to generate reproductive isolation via assortative mating, and this mate preferences may be highly efficient to maintaining isolation, thus the hypothesis of ecological speciation is supported. Finally, in this study, two alternative-phenotype lakes are described. It is suggested that the origin of the segregated alternative phenotypes in both lakes is a consequence of ecological traits divergences; however in one of the lakes the alternative phenotypes arose from a founder population, meanwhile in the second lake the alternative phenotypes may arose by the ecological adaptation of the forms in allopatry.

It is not the strongest of the species that survives or the most intelligent that survives. It is the one that is the most adaptable to change…



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DECLARATION

I hereby declare that the work on this thesis has not been submitted for another degree. It is entirely my own composition and that research described herein was carried out by me unless otherwise stated or acknowledged.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Species and Speciation

Current estimates of the number of species on our planet range from 8 to 14 million (IUCN, 2008). Understanding the mechanisms that generate and maintain this enormous diversity of species has been the focal point of the evolutionary biology.

Biologists have long accepted Darwin's concept of natural selection as the central explanation of adaptation and evolutionary change. Darwin in his seminal work "On the Origin of the Species" (Darwin, 1859) suggested that species are arbitrary constructs that not only evolve but also divide under the force of natural selection. Darwin's theory basically proposes that the evolution of adaptive novelty, defined as a discrete phenotypic trait that is new in composition or context of expression relative to established ancestral traits (see West-Eberhard, 2003a) under natural selection is a two step process: first variation, initiation of change and the origin of new forms; second, spread, which in Darwinian terms is the differential reproductive success which causes an increase in frequency. By 1935, the Modern Synthesis theory emerged with evolutionists like Dobzhansky who complemented Darwin's ideas by stressing the importance of reproductive isolating mechanisms as a set of traits that prevent gene flow between taxa. Subsequently, Mayr (1942) then proposed the biological species concept (BSC), which identifies species as groups of interbreeding individuals that are reproductively isolated from other groups thus representing independent units of evolution.

The modern theory of evolution endeavours to describe all processes that generate diversity, in particular speciation. Speciation is the process that explains the generation of two reproductively isolated populations, for which gene flow between the different taxa is usually absent. The most accepted and supported (by an abundance of empirical evidence, see Schliewen *et al.*, 1994; Coyne & Price, 2000; Coyne *et al.*, 2004) inference as to how speciation can occur is described by a scenario of allopatric speciation in which a geographical barrier, or some physical isolation mechanism, interrupts the gene flow between populations and separates some fraction of the population of a species. However an alternative conjecture: speciation in sympatry, has arisen, although much more subtle and complex, speciation is also possible in the absence of any physical isolation mechanism (Coyne, 2007), thus the split may be an evolutionary consequence of

interactions within the speciating population and therefore an adaptation (i.e. cross-generational change in phenotype frequencies involving gene frequency change due to selection on heritable variation in phenotypes) (Dieckmann *et al*., 2004).

Sympatric speciation was suggested by Darwin. He saw this process as an important engine of biological diversity, arguing that new species arose in sympatry to fill empty niches in the "polity of nature" (Darwin, 1859). Mayr implied that sympatric speciation involves the evolution of reproductive isolation within the average dispersal distance of a single individual. In this kind of speciation the initial restriction of gene flow is caused not by geography or distance, but by biological features of organisms (Mayr, 1963), although he considered that sympatric speciation was theoretically unlikely.

Sympatric speciation, however, faces two fundamental problems: the first is the antagonism between selection and recombination. As selection tries to split a population into two parts, it is counteracted by interbreeding that continually breaks up the evolving gene complexes that produce reproductive isolation. Thus sympatric speciation may occurs, if either close linkage between genes (i.e. alleles at different loci are found together more or less often than expected) is involved in reproductive isolation or if assortative mating does evolve, both of which reduce recombination. The second problem is coexistence. Sympatric speciation requires that populations develop sufficient ecological difference to coexist during and after the evolution of reproductive barriers. Therefore, reproductive isolation and the ability to coexist must evolve simultaneously (Coyne *et al*., 2004).

Darwin's idea of sympatric speciation was an important alternative to interpret speciation however, it was a bit vague. One century later Maynard-Smith (1966) presented a model of adaptive speciation (*sensu* Dieckmann *et al*., 2004) that enclosed the two key elements of a sympatric speciation process as we understand it today: ecological diversification and the emergence of reproductive isolation. The common scenarios in the occurrence of sympatric speciation involve disruptive selection that drives a population in two different directions at once. When natural selection is strong it can cause the population to divide into subpopulations, each specializing on a different resource. The most plausible forms of sympatric speciation, including disruptive selection on mate choice, involve an initial partitioning of ecological space followed by the evolution of mate discrimination. Speciation can occur if individuals either mate exclusively on the resource

they use (habitat isolation), or choose as mates only individuals using the same resources (sexual isolation) (Coyne, 2007).

1.2. Ecological Speciation

Ecological speciation is the process by which barriers to gene flow evolve between populations as a result of ecologically based divergent selection; that is when it acts in contrasting directions in the two populations favouring opposite, usually extreme, phenotypes within a single population, as occurs during sympatric speciation. Selection is ecological when it arises as a consequence of the interaction of individuals with their environment during resource acquisition (Rundle & Nosil, 2005).

The individual phenotype is defined as all aspects of an organism other than the genotype (West-Eberhard, 1989)**;** it is suggested as the object of natural selection (Mayr, 1947; 1963) rather than genotype, since natural selection does not act directly on mutations or genes and does not concern reproduction by genes themselves. To propagate differentially or spread within populations, genes depend on their ability to affect the reproduction of the bodies that contain them by affecting their phenotypes, following the selfish gene theory (Dawkins, 1976). Therefore, selection should be seen as acting on phenotypes and selectable variation means phenotypic variation, whether it has a genetic component or not (West-Eberhard, 2005a).

Thus, ecological speciation might come as a consequence of natural selection on morphological, physiological or behavioural traits (Schluter, 2001) with reproductive isolation evolving as a ultimate consequence of the divergent selection on these traits between different environments (Schluter & McPhail, 1992; Schluter, 2001).

The first stages of incipient ecologically driven speciation may involve divergence in trophic behaviour (e.g. dietary and habitat selection), usually followed by subsequent adaptive modifications in morphology (Skúlason *et al.*, 1999). Such ecological specialisation can potentially lead to the expression of stable alternative phenotypes which may be an initial step in subsequent incipient, adaptive sympatric speciation from a single gene pool (Rice & Hostert, 1993; Ackermann & Doebeli, 2004).

Ecological speciation may involve three main causes to divergent selection (Schluter, 2001)**:** differences between environments, for example, habitat structure, occupation of separate niches, climate, resources, and the suite of predators or competitors present (Schluter, 2000). Sexual selection, because it acts on traits directly involved in mate recognition (Boughman, 2001) and ecological interactions that are frequency dependent and mostly occur in sympatry, generating disruptive selection and then speciation (Rundle & Nosil, 2005). Ecological selection has been found in several species. For example, pea aphids (Via, 1999), *Rhagoletis* fruit flies (Linn *et al.*, 2003), *Timema* walking sticks (Nosil, 2004), *Littorina* snails (Rolan-Alvarez *et al.*, 1999), freshwater and marine sticklebacks, *Gasterosteus aculeatus* L. (Nagel & Schluter, 1998; Rundle *et al.*, 2000; Boughman, 2001; McKinnon *et al.*, 2004) which show pre-zygotic reproductive isolation caused by habitat divergence, ecological interactions and sexual selection and *Salvelinus alpinus* (L.) (Knudsen, 2006).

1.3. Origins of Diversification and Alternative phenotypes

In Maynard-Smith's model a species first evolves one or more new forms for specialisation in different habitats and subsequently assortative mating with respect to the habitat character. Novel forms have been defined as polymorphisms. This word was invented to refer to several forms at the same stage of development, however, the word has been used with strong focus on genotype-specific expression in ecological genetics (West-Eberhard, 2003a). In Mendelian genetics polymorphism refers to different allozymes whereas in ecological genetics it refers to different genotype-specific structural phenotypes maintained in the same population. Some insist that it implies only to morphology whereas others prefer a broader interpretation and include behaviour and physiology. Some insist that polymorphisms must be "genetically" determined and does not include continuous variation, but relatively sharply contrasted differences which either do not overlap or else give rise to a bimodal distribution (see Ford, 1945; Ford, 1966), while others include environmentally cued forms (Skúlason & Smith, 1995; Smith & Skúlason, 1996). Thus, the term polymorphism is of limited usefulness because its interpretation diverges amongst every sub-discipline in biology. Also, other terms have been created, depending on the nature of the expressed forms. For example, polyphenisms: irreversible environment-specific forms, most commonly morphological ones (Wakano & Whiteman, 2008; Takahashi & Parris, 2008; Karlsson & Johansson, 2008) and polyethisms, behavioural alternatives (Komdeur, 2006).

Henceforth, to avoid confusions the term "alternative phenotypes" will be used in place of polymorphism, polyphenisms, polyethisms or any alternative behavioural or physiological trait. Polymorphism will only be used for genetic analysis of restriction fragments. Alternative phenotypes are defined as different traits expressed in the same life stage and population, more frequently expressed than traits considered anomalies or mutations, and not simultaneously expressed in the same individual (West-Eberhard, 1989). Also, they may present a continuous variation or may exhibit contrasted differences, such as discrete phenotypes.

When the range of expressed characters is extended or when novel characters are expressed within a population, diversity increases. In some species individuals may exhibit different appearances, which will be retained during their entire life; these alternative phenotypes can be maintained in the same life stage in a single population (West-Eberhard, 2002). This developmental switch or mechanism of change producing alternative phenotypic expressions appears to be controlled by both genetic (allelic-switch) and environmental factors (Wakano & Whiteman, 2008) and the relative importance of these effects depends most likely on past and present selective environments as well as developmental constraints (West-Eberhard, 1989).

A genome input, like a mutation, leads to the production of a small phenotypic change in a single individual. Then owing to a fitness increase associated with the mutant genetic allele, the mutant gene and the associated phenotype increase in the population over subsequent generations (West-Eberhard, 2008). Thus, frequency of the trait will increase slowly (West-Eberhard, 2005a).

Alternatively, the origin and evolution of novelties can be facilitated by phenotypic accommodation (an individual's flexible responsiveness to external and internal environments) that is nongenetic adjustment among variable aspects of the phenotype following a novel input during development. Then, the novel phenotype may increase in frequency rapidly, within a single generation (West-Eberhard, 2005b).

Phenotypic plasticity plays the most important role in the origin of alternative phenotypes (West-Eberhard, 2005b). It is defined as the expression of multiple alternative phenotypes resulting from exposure to different environmental (internal and external)

conditions (West-Eberhard, 1989; Pigliucci, 2005). It is considered a trait since there is genetic variation in nature for plastic responses, therefore in a population different genotypes may show different reaction norms, i.e any trait may be more or less plastic. Phenotypic plasticity as a trait is thought to confer significant fitness advantage for organisms invading new habitats or living in highly heterogeneous or rapidly fluctuating environments (West-Eberhard, 1989; Scheiner, 1993; Via *et al.*, 1995; Schlichting, 2004). It is what makes possible the appearance of an environmentally induced novel phenotype. It allows the initial survival of organisms under a process of selection on the expression of such phenotype in the new environment. Such a process may end up 'fixing' (genetically assimilating) the novel phenotype by altering the shape of the genome response to the environmental input (reaction norm) (Pigliucci, 2005). Each alternative phenotype has a distinctive (or distinctively expressed) set of specific modifier genes, whose expression is ultimately regulated by a relatively simple cue (environmental, or allelic, or both) (West-Eberhard, 1989). Therefore, selection can act semi-independently upon alternative modes provided by the expression of one or more discrete phenotypes and thus has the potential to drive alternative phenotypes towards different evolutionary outcomes (West-Eberhard, 2003a). This effect is particularly evident where alternative phenotypes are expressed in sympatry (Schluter & McPhail, 1992) and where the expressed phenotypes have a strong functional significance (Adams & Huntingford, 2002b; West-Eberhard, 2005a; Schmidt *et al.*, 2006; Malaquias *et al.*, 2009). Thus examination of sympatric alternative phenotypes, amongst traits that have significant ecological importance for the organisms expressing those traits, has the potential to offer unique insights into the selective forces and evolutionary processes shaping change.

1.4. Ecological Factors that promote Phenotypic Diversity

Ecological interactions have been implicated in a number of speciation events in nature. For example, predation is a ubiquitous factor that influences the phenotypic variation in several groups of organisms (Jiggins *et al.*, 2001; Vamosi & Schluter, 2002). Within the fishes, predation has important ecological consequences, for instance, the presence of pike (*Esox lucius*) as a predator determines body morphology in crucian carp (*Carassius carassius*) which show enhanced escape locomotor performance and development of a deep-body in response to the predator (Domenici *et al.*, 2008). Moreover, environmental differences such as the influence of the physical characteristics of the habitat in the phenotypic variability of organisms are also considered of major importance.

Water flow and oxygen can influence directly the gill size, body shape and caudal fin shape in the African cyprinid *Barbus neumayeri* (Langerhans *et al.*, 2007). Spoljaric and Reimchen (2008) showed that water clarity may influence the degree of difference among males and females from the same population of three-spined sticklebacks. Their laboratory experiments showed that populations reared in a clear water habitat have greater sexual differentiation than those from deeply stained habitats. Furthermore, alternative phenotypic expressions are often related to segregation in habitat use, different benthic substratum habitats within lakes influence the existence of sympatric phenotypes as demonstrated by the three-spined sticklebacks caught in lava and mud habitats within four Icelandic lakes (Kristjansson *et al.*, 2002). In the lake Thingvallavatn, the three-spined sticklebacks that dwell in mud develop longer spines than the ones living in the lava habitat. This is thought to be because they experience higher predation pressure (Malmquist *et al.*, 1992). The mud dwelling fish also had longer gill rakers and generally feed on crustacean prey whereas the lava dwelling sticklebacks seems to be specialised chironomid feeders. Kristjansson (2005) also reported the influences of habitat differences in three-spined sticklebacks, he found that marine three-spined sticklebacks can change their morphology and armour characteristics extremely quickly when they are acclimatised in freshwater ponds.

Amongst the fishes, expression of alternative trophic phenotypes often involves differences in morphological characteristics used in the detection, capture or handling of prey items (Skúlason & Smith, 1995; Adams & Huntingford, 2002b). In lakes with sympatric phenotypes, the alternative phenotypes are typically very closely related and individuals can sometimes shift from one phenotype to another during a lifetime (Adams, 1999).

The expression of alternative trophic phenotypes is related to the level of plasticity that animals can have. Furthermore, morphological plasticity and behaviour can be a dichotomy between themselves: predominant plasticity of behaviour affects foraging efficiency and predominant morphological plasticity affects efficiency in handling the prey (Day & McPhail, 1996). Diet is considered one of the most important factors that produce alternative phenotypes in a population (Mittelbach *et al.*, 1992; Hegrenes, 2001). Several species have shown to be highly plastic in their trophic morphology as a response to the exposition to different prey items. For example, Ruehl and Dewitt (2007) using *Sciaenops ocellatus* species, the red drum, examined morphological and behavioural plasticity induced by durophagy (consumption of hard foods), they conducted feeding performance

trials to address the potential adaptive significance of diet-induced traits. Relative to soft foods, hard food induced a deeper head in the area of the pharyngeal mill, anterio-dorsally shifted eyes, and 8% heavier feeding muscles in juvenile *S. ocellatus*. Another example is the one demonstrated by Parsons and Robinson (2007) in the pumpkinseed sunfish (*Lepomis gibbosus)*. They reared young-of-year pumpkinseed sunfish from littoral and pelagic lake habitats each on a 'specialist diet' representing their native habitat-specific prey. The specialist diet induced divergent body forms that had a highest capture success of their native prey compared with generalist individuals. Furthermore, Walls *et al.* (1993) examined diet-dependent plasticity in head shape in larvae of the eastern long-toed salamander, *Ambystoma macrodactylum columbianum* by inducing variation. They found that larvae fed with tadpoles and brine shrimp (*Artemia sp.*) nauplii developed significantly broader, longer and deeper heads than did larvae that only ate brine shrimp nauplii. The ingestion of conspecifics, in addition to nauplii and tadpoles, significantly altered the inter-ocular width and the head depth, compared to larvae only fed nauplii and tadpoles.

In the nature, species of cichlids exhibit high degrees of trophic variation, related with dietary specializations. A cichlid from Cuatro Cienegas, Mexico, *Cichlasoma minckleyi*, presents two alternative phenotypes that differ in molarization, a papilliform phenotype with increased tooth measures and numbers and a molariform phenotype that maintains a relatively constant number of teeth as it produces teeth of progressively larger size (Whiteman *et al.*, 1996; Kassam *et al.*, 2003; Trapani, 2004; Hulsey *et al.*, 2005).

Dietary specializations amongst individuals of the same species are relatively common (Maerz *et al.*, 2006; Stuart *et al.*, 2006; Michaud *et al.*, 2008; Woo *et al.*, 2008). In most of the cases trophic morphological specializations have a functional significance for foraging prey detection, capture or handling (Smits *et al.*, 1996; Smith & Skúlason, 1996; Ferry-Graham *et al.*, 2002; Adams & Huntingford, 2002b; Hjelm *et al.*, 2003; Schmidt *et al.*, 2006; Januszkiewicz & Robinson, 2007; Knudsen *et al.*, 2008; Amundsen *et al.*, 2008; Malaquias *et al.*, 2009). Motta (2008) exemplifies this specialization in the nurse shark, *Ginglymostoma cirratum* that preys on benthic invertebrates and fish. The cranial morphology of this species exhibits a suite of structural and functional modifications that facilitates suction feeding. Suction is generated by the rapid depression of the buccopharyngeal floor by the coracoarcualis, coracohyoideus, and coracobranchiales muscles. Because the hyoid arch of *G. cirratum* is loosely connected to the mandible, contraction of the *rectus cervicis* muscle group can greatly depress the floor of the

buccopharyngeal cavity below the depressed mandible, resulting in large volumetric expansion. Maximum suction pressure does appear to be correlated with the rate of buccopharyngeal expansion.

The most evident cases of functional trophic specializations are the limnetic and benthic forms showed by postglacial fishes, where the former are better adapted to zooplankton consumption having a slender body, long, numerous, and densely spaced gillrakers, whereas the more robust benthic forms are specialised to feed on larger food items having less numerous, shorter and widely spaced gillrakers (Snorrason *et al.*, 1994). High trophic specialisation towards benthic or pelagic niches has also been observed in experimental feeding and growth studies of sympatric fish phenotypes (Schluter, 1995**;** Adams and Huntingford, 2002a; Klemetsen *et al.* 2006). The Limnetic phenotype of three-spined stickleback is less efficient in benthic feeding and the opposite is true for the benthic phenotype in pelagic feeding (Schluter, 1993).

Furthermore, some other vertebrates also exhibit alternative phenotypes with different degrees of phenotypic segregation (Smith & Skúlason, 1996; Relyea, 2001; Whiteman *et al.*, 2003). For instance phenotypes of the alpine newt, *Triturus alpestris*, differ in the hydrodynamics of prey capture. Paedomorphs water suck in water with prey items and expel it behind the mouth through gill bars; they show better feeding performance when they forage on aquatic crustaceans but are less successful when foraging on terrestrial invertebrates, meanwhile metamorphs expel water by the mouth as the gills are closed (Denoel *et al.*, 2004). Another amphibian that has distinct phenotypes is the larvae of salamanders *Hynobius retardus*, one of the phenotypes exhibits a broader head which has evolved to eat large, tough prey (Michimae & Wakahara, 2002). Also, Petranka *et al.* (1998) describe the existence of alternative colorations of eggs in *Ambystoma maculatum*, suggesting this alternative phenotypic expressions a response to *Rana sylvatica* tadpole predation.

1.5. Reproductive Isolation: Assortative Mating as a by-product

Ecology-driven reproductive isolation between populations may lead to ecological speciation when natural and/or sexual disruptive selection acts on morphological, physiological or behavioural traits (Mayr, 1947; Schluter, 2001). The models of

reproductive isolation are more commonly based on heterogeneous environments with two or more distinct niches, where a mechanism of adaptation to discrete resources is established. Thus speciation is caused by natural selection that favours phenotypes of both the extremes of the possible range (disruptive selection) and leads to reproductive isolations (Brigatti, 2006). For example, pre-zygotic isolation may evolve because mate choice happens to be based on traits that are the target of divergent natural selection (Servedio, 2004), or because divergent selection may favour shifts in mate choice criteria onto traits that are most conspicuous in each environment (Schluter & Price, 1993; Boughman, 2001).

Reproductive isolation as a by-product of divergent selection is certainly plausible, but evidence from evolution experiments in the laboratory give mixed results about how often it occurs. For example, Kilias *et al*. (1980) raised *Drosophila melanogaster* in either cold-dry-dark or warm-wet-light conditions and Dodd (1989) kept lines of *D. pseudobscura* on starch- or maltose-based media. Both studies found that some pre-zygotic isolation developed between flies from different environments, whereas almost no isolation evolved between different lines living in the same conditions. In contrast, Rundle (2003) found no effect of divergent selection on assortative mating between replicate *Drosophila* lines exposed to different environments. Studies of reproductive isolation from natural populations have demonstrated that traits under divergent natural selection are involved in reproductive isolation. For example, the Galapagos finches show disruptive selection in beak shape which determines diet (Schluter & Grant, 1984; Grant & Grant, 2008; Hendry *et al.*, 2009) and have additional effects on auditory mate recognition (Huber & Podos, 2006). Also, Jones *et al*. (2006) found that three-spined sticklebacks, from the River Tyne, Scotland show significant heterozygote deficit and cytonuclear disequilibrium in juveniles collected from sympatric sites. The authors suggested a potential contribution of temporal, spatial, and sexual pre-zygotic barriers to the observed reproductive isolation as well as post-zygotic selection against hybrid zygotes or fry.

In sympatry, the pre-zygotic key mechanism ensuring reproductive isolation over time is assortative mating. This mechanism can be selected per se, via reinforcement (Noor, 1999), or as a by product of specialisation (Rice, 1987). Therefore the strength of positive assortative mating and disruptive selection are the conditions that determine the resultant levels of genetic divergence and reproductive isolation in sympatric speciation (Gavrilets, 2006).

In fish, several examples have shown the importance of assortative mating. Extensive intra- and interspecific variation in male nuptial coloration and female mating preferences, in the absence of post-zygotic isolation between species of haplochromine cichlids of Lake Victoria, has inspired the hypothesis that sexual selection has been a driving force in the origin of this species flock. This hypothesis rests on the premise that the phenotypic traits that underlie behavioural reproductive isolation between sister species diverged under sexual selection within a species. In two closely related species of haplochromid cichlid, *Haplochromis nyererei* and the *Haplochromis ``zebra nyererei''*, males nuptial colouration trait is under directional sexual selection by female mate choice. This is a central cue in both interspecific (Seehausen & van Alphen, 1998) and intraspecific mate choice (Maan *et al.*, 2004), suggesting its importance in reproductive isolation. Visual early learning was also shown to mediate assortative shoaling preferences in zebra fish (Grünbaum *et al.*, 2007). The fish discriminate between shoals having different pigment pattern phenotypes and that early experience determines shoaling preference (Engeszer *et al.*, 2004). In a sympatric speciation scenario, for this fish, disruptive sexual selection on coloration may have initiated divergence of mating cues (Maan *et al.*, 2004). Furthermore, Vines and Schluter (2006) have shown that morphological traits are also relevant to mate assortatively. They found that given a choice, allopatric benthic-like females prefer benthic-like males and allopatric limnetic-like females prefer limnetic-like males, suggesting that mate preferences change readily as a consequence of ecological adaptation.

In addition, Rundle *et al.* (2000) showed that populations of three-spined sticklebacks from lakes in Coastal British Columbia, that evolved under different ecological conditions show strong reproductive isolation, whereas populations that evolved independently under similar ecological conditions lack isolation. In this species, there is good evidence of assortative mating. Mate preferences and mate choice in the three-spined sticklebacks are commonly based on multiple characters (Baker & Foster, 2002). Females mate choice is based on nuptial colouration pattern (Scott, 2004), nest site and structure (Blais *et al.*, 2004), courtship behaviour of the male (Ólafsdóttir *et al.*, 2006) , habitat choice (Vamosi & Schluter, 1999), and symmetry of male spines (Mazzi *et al.*, 2003). Mate choice is often based on body size and assortative mating may evolve between fishes of different size groups (McKinnon *et al.*, 2004). Some populations of sticklebacks present males that display preferences for different sizes of females (Albert & Schluter, 2004) and

in some other populations, females choose to mate assortatively by size (Rundle & Schluter, 1998).

The common observation of size-assortative mating in systems of sympatric phenotypes of freshwater fishes has important implications for models of speciation because it may indicate that divergence in nature is best described by single character models. Knudsen *et al* (2006) conclude that the evolution of assortative mating may be based directly on ecological traits induced by a profundal lifestyle of the small-sized profoundal phenotype of Arctic charr from Fjellfrøsvatn which is most likely under selection for heterochronic differences, notably paedomorphosis that could produce important traits for assortative mating.

Thus, assortative mating is required for the maintenance and increased degree of morphological divergence of alternative phenotypes within species (Johannesson *et al.*, 2008).

1.6. Postglacial Freshwater Fishes

The coexistence of alternative forms of freshwater fish, differing in traits that have a role in foraging, are now known to be relatively common in arctic areas where numerous lakes and rivers were formed as the ice cap retreated at the end of the last glacial epoch some 10,000-15,000 years ago (Schluter & McPhail, 1992; Wimberger, 1994; Skúlason & Smith, 1995; Skúlason *et al.*, 1999).

There is a robust and growing literature that demonstrates the expression of two or more discrete suites of alternative phenotypic traits that correlate with alternative foraging ecology. Amongst the whitefish, *Coregonus lavaretus*, some populations have two sympatric forms, differing in their habitat, ecology and morphology (Kahilainen & Ostbye, 2006). For example, the whitefish from six lakes of the St. John river basin (eastern Canada and northern Maine) have small body-size (dwarf) and large body-size (normal) ecotypes which differ primarily by traits related to trophic specialization within lakes; they show significant but variable genetic divergence. The reproductive isolation reached between dwarf and normal whitefish ecotypes appears to be driven by the potential for occupying distinct trophic niches and, thus, by the same selective forces driving tropic

specialization in each lake (Lu & Bernatchez, 1999). Within Coregonine fishes, local genetic differentiation is often coupled with eco-phenotypic diversification. Gill raker alternative phenotypes, depth-related habitat preference and reproductive behaviour are considered as phenotypic traits with probable adaptive value contributing to the niche expansion of ciscoes, *Coregonus artedi* (Turgeon & Bernatchez, 2003).

In sympatry the coexistence of two or more discrete intralacustrine phenotypes may have two alternative origins. Alternative phenotypes can be either originated by intralacustrine divergence of one founder population (sympatry) (Hindar *et al.*, 1986; Bodaly *et al.*, 1992; Foote *et al.*, 1992) or, in some cases, patterns of genetic diversity indicate multiple invasions of the forms representing different lineages (Robinson *et al.*, 2000b) (Bernatchez & Dodson, 1990; Pigeon *et al.*, 1997; Skúlason *et al.*, 1999; Alekseyev *et al.*, 2002). In whitefish, for example, sympatric pairs coexisting in three lakes from the southern Yukon represent genetically distinct reproductive units with a polyphyletic origin whereby each of them have been expressed independently more than once. In the two lakes the existence of sympatric pairs is best explained by the secondary contact of two monophyletic whitefish groups that evolved in allopatry during the last glaciation events (Bernatchez & Dodson, 1990; Bernatchez *et al.*, 1996)

Moreover, morphological variation driven by phenotypic plasticity has been demonstrated in another postglacial fish, Arctic charr, *Salvelinus alpinus* (L.) (Snorrason *et al.*, 1994; Skúlason & Smith, 1995; Smith & Skúlason, 1996; Adams *et al.*, 1998; Alexander & Adams, 2000; Klemetsen *et al.*, 2002; Alekseyev *et al.*, 2002; Adams *et al.*, 2003a; Andersson *et al.*, 2005; Power *et al.*, 2005). This species is the most northerly distributed freshwater fish having and Holarctic distribution (Skúlason *et al.*, 1999; Wilson *et al.*, 2004). The Arctic charr has been heavily influenced in their zoogeography and genetic structure by Pleistocene glaciations processes (Wilson *et al.*, 1996; Wilson *et al.*, 2004). In the British Isles the Arctic charr originates from a single "Atlantic" lineage (Brunner *et al.*, 2001), this has given rise to speculations about the sympatric variation of several phenotypes within lakes across Scotland.

The Arctic charr is a species that exhibits a very high degree of phenotypic plasticity and frequently forms subgroups that coexist and exploit a relatively narrow range of prey among several types of available prey and differ in aspects like body-size and their feeding apparatus: head size and shape, jaw length, gillrakers number, eye diameter, as well as the

option for foraging habitats and their feeding behaviour (Nordeng, 1983; Hindar & Jonsson, 1993; Adams & Huntingford, 2002a; Adams *et al.*, 2003a), also charr phenotypes differ in life history traits (Eiríksson *et al.*, 1999).

The morphology of Arctic charr from some populations seems to reflect their resource partitioning. Specialised phenotypes may be able to grow better and retain higher densities than intermediate forms (Jonsson & Jonsson, 2001). Also charr phenotypes appear to have developed under intense specific competition where extreme morphologies feed more successfully than intermediate phenotypes (Hindar & Jonsson, 1982).

The Arctic charr sympatric foraging specialisms most frequently comprise individuals specialising in preying upon plankton, macro-invertebrate benthos or fish accompanied by discrete morphological variation in functionally significant traits (Eiríksson *et al.*, 1999). Although the functional significance of many expressed alternative phenotypes is difficult to prove, a large number of described alternative phenotypes is the result of variation in the anatomy of the feeding apparatus (trophic alternative phenotype) clearly indicating a functional role in foraging (Malmquist *et al.*, 1992; Adams *et al.*, 2003b). Adams and Huntingford (2002b) showed the functional importance of the differences in the form of the mouth suggesting that these are associated with necessities of foraging and preferences in diet. When offered an option between a typical benthic prey and a pelagic prey, näive Arctic charr individuals from benthic habitats were more disposed to feed on benthic prey, while those from pelagic origin fed on pelagic prey.

Individuals of this species that come from benthic phenotypes have a wider mouth in relation to the body longitude in contrast to those that come from the pelagic phenotypes. Pelagic individuals have a fusiform body, with brilliant coloration while they spawn, they have a slight construction of the jaw, terminal mouth, short pectoral fins, and long and dense gills; in the field they feed exclusively on zooplankton. Whereas the benthivorous phenotype frequently has cryptic colours, they have a more robust head and body, sub-terminal mouth, large pectoral fins, and relatively short and spaced gills and they feed on benthic macro-spineless invertebrates (Malmquist *et al.*, 1992; Adams & Huntingford, 2002a).

Some phenotypes use the same habitat but they rarely overlap with regard to diet, thus behavioural and morphological differences may be based on the preferences of prey,

instead of being competitive or predatory interactions. This may be a primary mechanism to maintain ecological separation. For example, in the case of the limnetic and benthic morphs from Thingvallavatn, the two types of morphs exhibit very different use of food and are partially segregated in habitat (Malmquist *et al.*, 1992).

The potential trophic niche of an individual is ultimately determined by the limits of its behavioural, morphological and physiological abilities related with its feeding (Schluter, 1993). The poor efficiency of benthic form charr feeding on zooplankton and its general reaction indifference to this prey can reflect morphological and behavioural limitations. As benthic charr only rarely consume zooplankton in their natural environment, absence of zooplankton from their diet should be related to morphological limitations. Consequently, trophic segregation observed between benthic and limnetic types could be determined through natural selection and it could provide a foraging efficiency that affects fitness of each phenotype (Malmquist *et al.*, 1992; Snorrason *et al.*, 1994; Kassam *et al.*, 2004)

Heterochrony is defined as an evolutionary change in the timing of the expression of a phenotype trait (e.g. size), that transfers expression of the trait from one life stage or behavioural or physiological phase to another (West-Eberhard, 2003a). This characteristic has been suggested as one potential functional mechanism through which alternative phenotypes in Arctic charr may evolve (Adams & Huntingford, 2002a). Due to the feeding opportunities in the various habitats exploited, the phenotypes show significant variation in size, which strongly affects resources use by the fish (Grünbaum *et al.*, 2007). Also differences in age at sexual maturity cause some variation in adult sizes among phenotypes. Thus early maturing phenotypes become smaller than sympatric, late maturing ones (Hindar & Jonsson, 1993). Body size is probably the most important phenotypic trait in the life history, habitat use and evolution of the *Salvelinus* species and is often an important trait in assortative mating of sympatric pairs of postglacial fishes (Boughman, 2001; Wenrick Boughman *et al.*, 2005).

The extensive adaptive radiation in phenotypic and genetic diversity within the *Salvelinus* complex has been widely described. Although there are doubts about the integrity of the species, current thinking is that *S. alpinus* is still the single species status (Adams and Maitland, 2006).

Another important phenotypically plastic postglacial species is the three-spined stickleback, *Gasterosteus aculeatus* L. This species, also exhibit alternative phenotypic expressions through its northern distribution (Bell & Foster, 1994; Östlund-Nilsson & Mayer, 2007). There are strong relationships among foraging behaviour in a specific habitat, proportion of capture and morphology in the group (Bentzen & McPhail, 1984; Schluter & McPhail, 1992; McPhail, 1992; Day & McPhail, 1996). A relationship between foraging and morphology of fishes that strongly correspond to differences in growth and diet of limnetic and benthic groups are commonly described. The limnetic form is better adapted to zooplankton consumption having a slender body, long, numerous, and densely spaced gillrakers, whereas the more robust benthic form is specialized to larger food items having less numerous, shorter and widely spaced gillrakers and bigger mouth (Foster *et al.*, 1992; McPhail, 1992; Schluter, 1993; Bell & Foster, 1994; Cresko & Baker, 1996; Baker *et al.*, 2005). The limnetic phenotype of the three-spined stickleback is less efficient in benthic feeding, while the opposite is true for the benthic phenotype in pelagic feeding (Schluter, 1993).

Three-spined sticklebacks also show phenotypic segregation in reproductive life-history traits. For example in Benka Lake, Alaska females of the two ecotypes show difference in reproductive allocation, with benthic females producing fewer, larger eggs (Baker *et al.*, 2005). Also, it has been reported that sticklebacks sympatric pairs may show differences in signalling traits such as male nuptial coloration (Albert *et al.*, 2007), armour apparatus (pelvic spines reduction due to predators presence) (Klepaker & Ostbye, 2008), learning and orientation (Girvan & Braithwaite, 1998; Girvan & Braithwaite, 2000), body size, morphology and symmetry (Moodie & Moodie, 1996; Nagel & Schluter, 1998), nest-site (Mori, 1994) and habitat use (Schluter, 1993).

Divergence in size is considered a common feature of ecological divergence in sticklebacks, and several studies have found size assortative mating between ecologically differentiated stickleback populations (Nagel & Schluter 1998; Ishikawa & Mori 2000; McKinnon *et al*. 2004; Vines and Schluter, 2006).

1.7. Postglacial Freshwater Fishes as Model Species in the present Study

Sticklebacks and Arctic charr were selected for this study because they exhibit high levels of phenotypic plasticity, have simple husbandry and a wealth of data exist on the ecology and evolutionary biology of the species (Bell & Foster, 1994; Schluter, 2000; Adams and Huntingford, 2002 a, b; Klemetsen *et al.* 2006; Vamosi, 2003; Östlund-Nilsson & Mayer, 2007). The stickleback has a reasonably short generation time, exhibit elaborate behaviour and occupy counted isolated habitats in which they have evolved extraordinary phenotypic diversity (Schluter & McPhail, 1992). As mentioned earlier in this text, both species show divergence in many traits, including body size (Nagel & Schluter, 1998; Jonsson and Jonsson, 2001; Adams *et al.* 2003), body shape (Walker, 1997; Kristjansson, 2005), trophic characters (Day & McPhail, 1996; Wund *et al.*, 2008; Adams and Huntingford, 2002 a; Alekseyev *et al.*, 2002), antipredator traits (Reimchen, 2000), male reproductive characters (Albert & Schluter, 2004; Albert *et al.*, 2007) and swimming performance (Alvarez & Metcalfe, 2005).

Here, morphological characteristics are the main focus used to describe the plasticity of these species and the importance of their phenotypic divergence. To evaluate this important trait the very modern landmark-based geometric morphometrics technique was applied.

1.8. Landmark-based Geometric-Morphometrics

Geometric morphometrics is an alternative tool to the traditional approach that allows the study of shape variation and its covariation with other variables (Bookstein, 1991). Here geometric morphometrics techniques are based on the collection of two dimensional coordinates of biologically definable landmarks (Adams *et al.*, 2004), that are most convenient to describe the expected pattern of shape variation than traditional linear measurements (Rohlf, 1990).

Effects of variation in position, orientation, and scale of the specimens are present in the raw coordinates; therefore this variation is mathematically removed prior to the analysis of such variables. To remove the non-shape variation a superimposition method must be used by overlaying configurations of landmarks according to some optimisation criterion (Bookstein, 1991). The superimposition method applied here is the Generalised

Procrustes Analysis (GPA: called Generalised Least Squares, GLS, in the earlier literature) which is one of the most used methods, because it analyses shape with procedures based on Kendall's shape space which have the best statistical power, the lowest mean-squared error, and impose minimal constraints on the patterns of variation that can be detected (Rohlf, 1999; Rohlf, 2000).

GPA superimposes landmark configurations using least-squares estimates for translation and rotation parameters (Rohlf & Slice, 1990). First, the centroid of each landmark configuration (shape) is calculated as the position of the averaged coordinates of the landmarks then each centroid is translated to the origin. Second, the configuration is scaled to a common unit by dividing by centroid size (Bookstein, 1991). The centroid size is a size-measure computed as the square root of the summed squared Euclidean distances from each landmark to the specimen's centroid. The final step is the optimal rotation of the configurations to minimize the squared differences between corresponding landmarks (Rohlf & Slice, 1990). When minimized simply by rotation, this quantity is called the Partial Procrustes distance (ρ). After rotation to partial Procrustes superimposition (Fig. 1.1a), the square root of the sum of the squared differences between the coordinates of corresponding landmarks can be further reduced by rescaling the target to centroid size of cos (ρ). Configurations that satisfy this condition are said to be in full Procrustes superimposition on the reference: and the resulting distance between shapes is the full Procrustes distance. The set of shapes in full Procrustes superimposition comprises a hypersphere of radius one-half, inside the hemisphere of shape in partial Procrustes superimposition, and tangent to the larger hemisphere at the reference. This smaller, inner hypersphere is Kendall's shape space, Fig. 1.1b (Zelditch *et al.*, 2004).

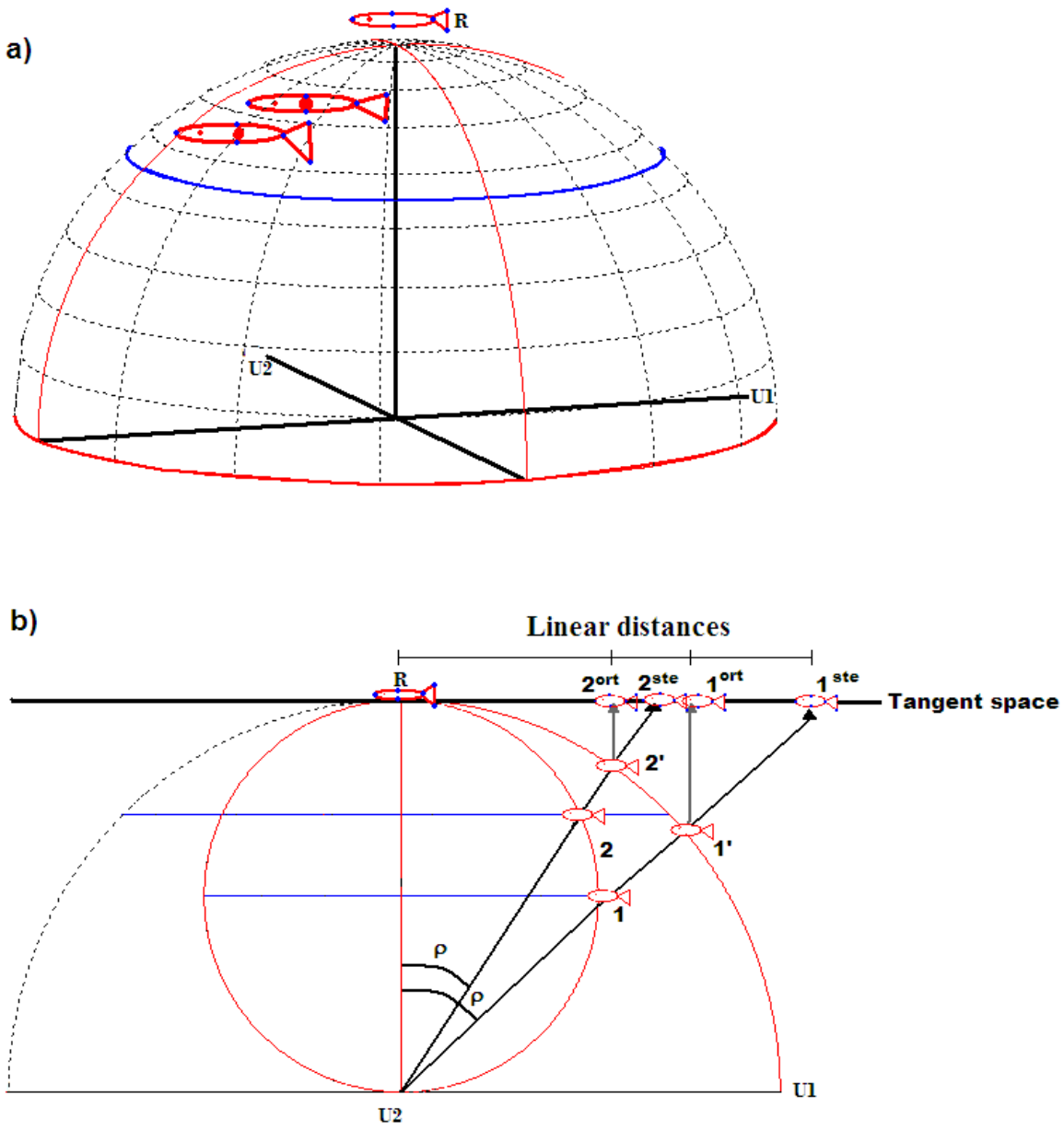

Figure 1.1 Shape spaces a) space of centred and aligned shapes (red fish) scaled to unit centroid size b) Section through the hemisphere of aligned shapes space and the inner circle is a section through Kendall's shape space of centred and aligned shapes scaled to cos (ρ). The plane is tangent to the sphere and the hemisphere at the point of reference shape. The configuration at points 1 and 2 represent a fish shape in Kendall's shape space; 1' and 2' are the same fish shapes scale to unit centroid size. 1^{ort} and 2^{ort} are the orthogonal projections of 1 and 2 onto the tangent plane respectively. 1^{ste} and 2^{ste} are the stereographic projection of 1 and 2 onto the tangent plane respectively. U1 and U2 refer to uniform component. R represents the mean shape or reference. Landmarks are indicated in blue points.

The alignment of all the specimens allows the estimation of the mean shape also called reference or tangent configuration (R in Fig. 1.1b) because it is the configuration of landmarks that corresponds to the point of tangency between the exact non-linear Kendall's shape space (Kendall, 1984; Slice, 2007) and the approximated tangent space (Fig. 1.1b) in which the linear multivariate statistical analyses are performed (Rohlf, 1999). In this tangent space, distances between specimens pairs (represented by points) approximate the Procrustes distances between the corresponding pairs of landmark configurations.

A complementary technique called Thin Plate Spline (TPS) is applied to look for patterns in shape change. This method can be used to map the deformation in shape from one object to another (Bookstein, 1991). Differences in shape represented in this fashion are transformation grids, where one object is deformed or "warped" into another. This method models shape changes as deformations, by fitting an interpolation function to the aligned landmark coordinates of each specimen against the reference configuration. Differences in shape among the specimens and the reference configuration, fitted by the thin plate spline function, are expressed as a bending energy matrix, where the eigenvectors are denominated principal warps which eigenvalues are associated to the spatial scale of shape change (Rohlf *et al.*, 1996). The projection of the aligned specimens onto the principal warps yield to the matrix of partial warp scores. The partial warps are the new shape variables that can be analysed by conventional methods of multivariate statistics because they are simply linear combinations of the difference between each specimen and the reference configuration (Rohlf *et al.*, 1996).

The shape variation modelled by thin-plate spline technique is decomposed into the partial warp scores (non-uniform component) and the uniform shape components that represent shape changes that can be described by an infinite scale stretching or shearing (Rohlf & Bookstein, 2003). The parameters describing these deformations, are treated as multivariate data representing shape in which conventional multivariate analyses are performed (Adams & Rohlf, 2000; Costa & Cataudella, 2007; Langerhans *et al.*, 2007; Michaud *et al.*, 2008; Aguirre *et al.*, 2008).

Here, Relative Warp Analysis is performed to assess localized shape changes among morphologically distinct groups. The relative warps are the principal components of the partial-warp scores matrix (Rohlf & Marcus, 1993).

The average configuration of landmarks is used as the reference configuration. The relative warps are computed with the scaling option α=0, that weights all landmarks equally (Rohlf *et al.*, 1996), both non-uniform and uniform components are included in the analysis. Significance of the fish shape differences are assessed by analysis of variance of the relative warp scores. The results are visualised directly on fish shape by regressing the partial warps and uniform components onto each relative warp (Rohlf *et al.*, 1996).

The relative warp analysis and computation of the centroid size and partial-warp scores is done by using the tpsRelw program, version 1.45 (Rohlf, 2007). Regressions between partial warps and relative warps are computed with tpsRegr program version 1.31 (Rohlf, 2006b). All further statistical analyses of shape are performed with the SPSS 13 package.

In the present study the analysis of shape in individuals of postglacial species such as the Arctic charr and the three-spined sticklebacks is relevant to describe morphological divergence among alternative phenotypes within populations, where phenotypic plasticity plays a fundamental role. The study of divergent plastic trait responses and their fitness consequences in freshwater fishes of postglacial lakes is significant because they show considerable phenotypic variation in the form of trophic or resource alternative-phenotypes along an ecological gradient often bounded by littoral and pelagic habitats. Phenotypic plasticity is likely to have profound macroevolutionary consequences (Scheiner 1993; Schlichting and Pigliucci 1998; Pigliucci 2001, 2005, 2006; Parsons and Robinson 2006), yet few attempts have been made to empirically address the processes by which plasticity might influence phenotypic evolution (West-Eberhard, 2003; Pigliucci, 2005).

Over the past 2 decades West-Eberhard (1989; 2003; 2005; 2009) has dedicated time to review the additional importance of plasticity as a diversifying factor in evolution, a factor contributing to the origin of novel traits and to altered directions of change. She has described a model that first: outlines the nature of plasticity and its special relationship to natural selection and second: shows how phenotypic plasticity may act to facilitate and accelerate three major processes in evolution: the origin of novelty, speciation, and macroevolution.

1.9. The role of developmental phenotypic plasticity in the origin of divergence and speciation (West-Eberhard model)

West-Eberhard (1989) suggested that extensive divergence via intra-specific alternative phenotypes may occur prior to the assortative mating or reproductive isolation of distinctive forms. Also, that this divergence can involve condition-sensitive or environmentally cued (not only allelic-switch, genetically) alternatives and that environmentally cued traits facilitate sympatric speciation.

In 2003, she summarised the steps of an alternative phenotype hypothesis that could apply to sympatric speciation (West-Eberhard, 2003a):

1. Establishment of divergent, discrete, or bimodally distributed complex alternative phenotypes in both sexes.

    a. A novel input occurs which affects one (if a mutation) or possibly more (if environmental) individuals.
    b. Phenotypic accommodation: individuals developmentally responsive to the novel input immediately express a novel phenotype.
    c. Initial spread: the novel phenotype may increase in frequency rapidly within a single generation if it is due to an environmental effect that happens to be common or ubiquitous. Alternatively, if it is due to a positively selected mutation or is a side effect of a trait under positive selection, the increase in frequency of the trait may require many generations.

2. Incidental assortative mating by males and females of like phenotype due to parallel alternative tactics or traits in both sexes (mating time or place, size matching, habitat similarity)

3. Incidental accumulation of phenotype-specific genetic divergence in alleles that affect regulation and form, as an effect of assortative mating between individuals of like phenotype and genotype.

4. Adaptive assortative mating due to selection (usually on females) to increase the genetic quality of offspring by choice of ecologically compatible mates that express a parallel phenotype.

5. Mutual acceleration of bidirectional divergence (phenotypic and genetic) in regulation and form, further accelerated by character release and bidirectional sexual selection.

6. Lineage-specific predominance or fixation of a single alternative.

7. Further increased premium on assortative mating and reproductive isolation (speciation) due to increased genetic and phenotypic divergence of the fixed form.

1.10. Overall aims and Thesis structure

The main focus of the present study is to elucidate how phenotypic variation contributes to speciation. Although there is growing evidence that alternative phenotypes maybe important intermediate stages in the route to full speciation (Smith & Skúlason, 1996; Schluter, 2001), the origin of alternative phenotypic expressions it is not clear. With this background the work described in this thesis was designed to answer several questions, following the West-Eberhard (2003a) alternative phenotype hypothesis model that could apply to sympatric speciation.

1. Do environmental inputs (i.e. physical characteristics of habitat) modulate phenotype expression through developmental plasticity?

The general aim of chapter 2 is to test the hypothesis that external surrounding environment can directly induce morphological variation through phenotypic plasticity in three-spined sticklebacks (*Gasterosteus aculeatus*) in a laboratory controlled experiment. Here, the first step of the alternative phenotype hypothesis model is supported.

2. Do morphology and discrete prey types promote dietary specialization in Arctic charr?

Chapter 3 tests the degree to which individuals from a single population exhibit foraging specialisations and the extent to which variations in morphology determine prey choice in individuals exposed to alternative prey. Here the dietary specialisation is considered as a step to the reinforcement of morphological divergence (phenotype fixation).

3. Are assortative mating choices based on expressed plastic phenotypic traits in three-spined Sticklebacks?

The goal of chapter 4 is to present an example of assortative mating driven by phenotypic plasticity. Here the step 1 of the model is supported again with a different environmental input (i.e. Diet). Specifically, first I test whether diet itself acts as the immediate mechanism to induce changes in body shape and trophic morphology in Sticklebacks. Secondly, I tested if body morphology is a proximate cause to assortative mating (steps 2-4 of the model).

4. Ecological, morphological and genetic evidence of alternative evolutionary origins in Arctic charr (*Salvelinus alpinus*) from two polymorphic systems in Scotland.

Here, in chapter 5, alternative origins of sympatric alternative-phenotypes in natural systems from two Scottish lakes are described to address questions relating to the proximate status and evolutionary origin of these phenotypes. Specifically five hypotheses are tested: the phenotypes in each lake 1) represent ecologically distinct units, 2) differ in functionally significant morphological characteristics, 3) exhibit different life history traits, 4) represent genetically distinct units, and 5) show similar patterns of evolutionary divergence. One bimodal population represents the last two steps in the model of sympatric speciation; meanwhile the second represents the alternative origin of sympatric phenotypes: multiple invasions.

5. Variation in scale shape amongst alternative sympatric phenotypes of Arctic charr from two lakes in Scotland.

In chapter 6, the main objective is to use landmark based geometric morphometrics to describe shape differences in fish scales between the two intralacustrine alternative phenotypes from Loch Awe and Loch Tay.

CHAPTER 2. HABITAT COMPLEXITY MODULATES PHENOTYPE EXPRESSION THROUGH DEVELOPMENTAL PLASTICITY IN THE THREE-SPINED STICKLEBACK *GASTEROSTEUS ACULEATUS*

*** Note: This chapter will be submitted as a manuscript to the Biological Journal of the Linnean society.**

2.1. Introduction

The development of diversity and the establishment of reproductive isolation are the two important elements required for speciation occurs (Mayr, 1963). Diversity in a population is increased when the range of expressed characters is extended or when novel characters are expressed. New characters or suites of expressed characters can display as alternative phenotypes within a single species and are particularly common amongst the fishes. Trophic alternative phenotypes (trophic polymorphism *sensu* Smith & Skúlason, 1996) with a functional significance for feeding have been commonly reported for cichlids species (Wimberger, 1992; Stauffer & Gray, 2004; Trapani, 2004; Kidd *et al.*, 2006; Swanson *et al.*, 2007), for salmonids (Adams *et al.*, 1998; Jonsson & Jonsson, 2001; Alekseyev *et al.*, 2002; Kahilainen & Ostbye, 2006; Whiteley, 2007; Bertrand *et al.*, 2008; Amundsen *et al.*, 2008; Noakes, 2008) and for sticklebacks (McPhail, 1992; Larson & Mcintire, 1993; Day & McPhail, 1996; Smith & Skúlason, 1996; Aguirre *et al.*, 2008).

The origin of alternative phenotypes is often thought to originate from ontogenetic processes, specifically phenotypic plasticity (West-Eberhard, 2003b). Phenotypic plasticity is the expression of multiple alternative phenotypes resulting from exposure to different environmental (internal and external) conditions. Phenotypic plasticity as trait is thought to confer significant fitness advantage for organisms invading new habitats or living in highly heterogeneous or rapidly fluctuating environments (West-Eberhard, 1989; Scheiner, 1993; Via *et al.*, 1995). Phenotypic plasticity has been demonstrated in a number of fish species (Smith & Skúlason, 1996; Alexander & Adams, 2000; Langerhans *et al.*, 2003; Adams *et al.*, 2003b; Baker *et al.*, 2005; Andersson *et al.*, 2005; Power *et al.*, 2005; Ruehl & Dewitt, 2005) and also has been reported in other vertebrates (Smith & Skúlason, 1996; Petranka *et al.*, 1998; Michimae & Wakahara, 2002; Relyea, 2002; Whiteman *et al.*, 2003; Denoel *et al.*, 2004).

The underlying drivers resulting in the expression of discrete alternative phenotypes have been examined by a number of studies (Meyer, 1987; Wimberger, 1992; Walls *et al.*, 1993; Winemiller, 1995; Smith & Skúlason, 1996; Swanson *et al.*, 2007; Michaud *et al.*, 2008; Amundsen *et al.*, 2008). Studies suggest antipredator behaviour as a cause of discrete phenotypic variation (Dewitt *et al.*, 1999; Reimchen, 2000; Laurila *et al.*, 2002), some birds such as orange crowned-warblers (*Vermivora celata*) show predator-induced plasticity in nest site (Peluc *et al.*, 2008). Diet and foraging have been implicated in the expression of coexisting benthic and limnetic foraging phenotypic specialists of three-spined sticklebacks (McPhail, 1992) and Arctic charr (Skúlason *et al.*, 1999).

Phenotypic alternative expressions often appear to be related to segregation in habitat use. For example different benthic substratum within lakes was correlated with the existence of sympatric phenotypes as demonstrated by sticklebacks caught in lava and mud habitat within four Icelandic lakes (Kristjansson *et al.*, 2002). In Thingvallavatn, three-spined sticklebacks living on mud substrates show longer spines because of predator defence (Malmquist *et al.*, 1992). Three-spined sticklebacks are known to exhibit plastic responses when exposed to different diets (Day & McPhail, 1996). In the wild, sticklebacks have different morphologies in different habitats (Day & McPhail, 1996; Walker, 1997; Nagel & Schluter, 1998; Reimchen, 2000; Albert & Schluter, 2004; Alvarez & Metcalfe, 2005; Kristjansson, 2005; Albert *et al.*, 2007; Wund *et al.*, 2008).

2.2. Aims

The goal of this chapter is to test the hypothesis that physical characteristics of the habitat can directly induce morphological variation through phenotypic plasticity in three-spined sticklebacks in a laboratory controlled experiment.

2.3. Methodology

2.3.2. *Fish sampling and holding conditions*

One hundred and twenty freshwater three-spined stickleback fry (approximately one month post hatching old, 18-15mm standard length) were caught by dip netting, from a pond in the Endrick River catchment, Stirlingshire, Scotland (56°3' N; 004°21' W) during summer. These were transported to rearing facilities at the Scottish Centre for Ecology and

the Natural Environment (SCENE), Glasgow University, Loch Lomond. Fifteen fish were randomly assigned to each of eight 21 litre holding tanks. Fish were divided into two treatment groups with four replicates. One treatment comprised tanks with a pea gravel substratum (5-10 mm), designated as the "simple habitat". The other treatment was designated to be the "complex habitat", thus, the aquaria contained large rocks (25-45mm) with significant interstitial spaces between them, and synthetic macrophytes in addition to a pea gravel substratum (5-10 mm). Water temperature was held at ambient Loch Lomond temperature for the duration of the experiment. Fish in all tanks were fed two times daily to satiation with defrosted chironomid larvae for the seventeen-week duration of the experiment.

2.3.3. *Morphological analysis*

At the beginning of the experiment fish were anaesthetised with benzocaine, and the left side of each fish was photographed digitally with a Nikon Coolpix 885 camera fixed to a camera stand and illuminated with blue light. A second batch of photographs were taken (as above) seventeen weeks after the experiments started.

The overall body shape was quantified using landmark-based geometric morphometrics analyses. Twenty landmarks were digitised on each image (see Fig. 2.1) using the tpsDig2.1 software (Rohlf, 2006a).

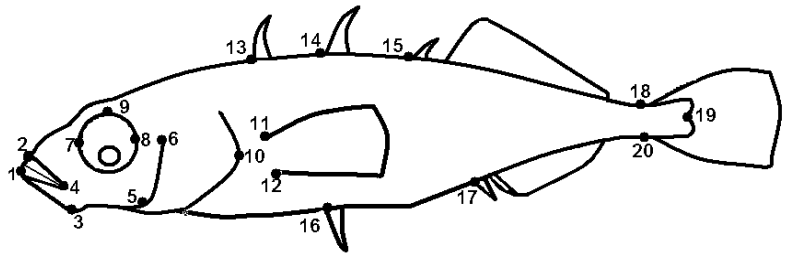

Fig. 2.1 Location of 20 anatomical landmarks collected from the left side of each specimen.

Landmarks configurations for each specimen were aligned, translated, rotated and scaled to a unit centroid size by the Generalized Procrustes Analysis (GPA) using the mean shape of all the images as starting form (Rohlf & Slice, 1990). Thereafter the TWOGroup6 program from the IMP series (Sheets, 2003) was use to performed a Goodall's F-test (Goodall, 1991) to determine mean fish shape differences between the two habitat groups.

The overall between-treatments shape variation was explored with a relative warp analysis (similar to Principal Component Analysis for morphometric data) using the TPSRelw software (Rohlf, 2007). Shape variation was quantified in individuals as deformations from the pooled mean shape i.e. reference or tangent configuration (Rohlf *et al.*, 1996).

To determine habitat effects the relative warps scores were analysed using one-way ANOVA. Thin plate splines were used to describe graphically the main changes in fish body shape.

2.4. Results

Sixty three mortalities were recorded between weeks one and seventeen (20 from the simple habitat and 43 from the complex habitat).

Average shapes of fish from each habitat treatment (simple and complex) were calculated. At the beginning of the experiment (august) there was no difference in the morphology of fish between habitat treatments (Goodall's $F_{20,2940} = 1.01$; $p= 0.4$).

After 17 weeks exposure to the two habitat conditions fish were not significantly different in centroid size ($F_{1,35}=0.422$; $p = 0.5$), however, there were significant differences in morphology (Goodall's $F_{36,3024}=3.6$; $p< 0.0001$). The first four principal components of the Relative Warps Analysis together explain 61.2% of the total variability in shape. However, all the significant variation between treatments occurs in the second relative warp (Table 2.1).

Table 2.1 F tests of the relative warps scores and percentage of explained variation.

Relative Warp	% of explained variation	d.f.	F	p
RW1	19.8	1, 84	0.04	0.8
RW2	16.5	1, 84	17.8	0.0001
RW3	14.1	1, 84	1.01	0.3
RW4	10.6	1, 84	0.9	0.3

The variation along the second relative warp describes the variation in both head and body shapes. Figure 2.2 depicts the mean and standard error of the scores for each treatment, the mean shapes are visualised as splines. Specimens with a low RW2 score are elongated and thin in the body, whereas, those with a high RW2 scores are broader and deeper. There is significant variation in head shape; individuals with low RW2 scores have smaller and shorter head and narrower mouths, more robust jaws and a more terminal mouth.

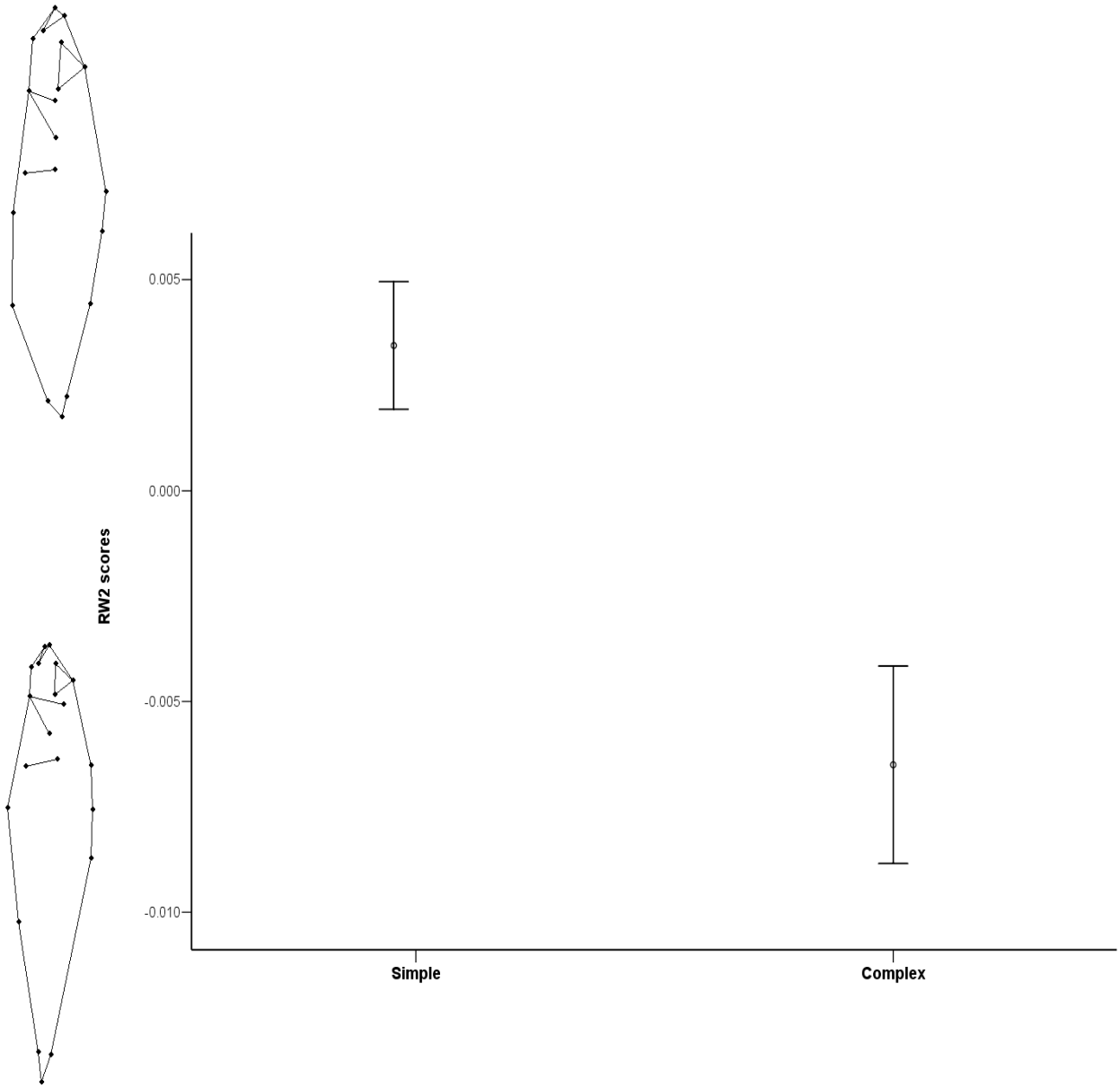

Fig. 2.2 Mean ±S.E second relative warp scores for sticklebacks from each of the two treatments. Splines of the shape of the more extreme scores and mean shapes of fish from each habitat treatment are shown. Links between landmarks are drawn to facilitate the visualization.

Fish reared in the complex habitat have their dorsal spines closer to their heads and the third spine is more distant from the caudal peduncle which is reduced. The middle spine is positioned along the same vertical axis as the pelvic spine. In contrast, fish exposed to the simple environment showed a longer distance between the superior part of the eye and the first dorsal spine, the three spines are more posterior, almost located in the posterior half of the body, the middle spine is not aligned with the pelvic spine and in general the caudal part of the body is shortened but broader, Fig. 2.3. *Post-hoc* tests carried out between tanks within treatment showed no evidence of a tank effect within the simple habitat treatment ($F=1.0$; $p=0.4$) nor within the complex habitat treatment ($F=2.2$; $p=0.1$).

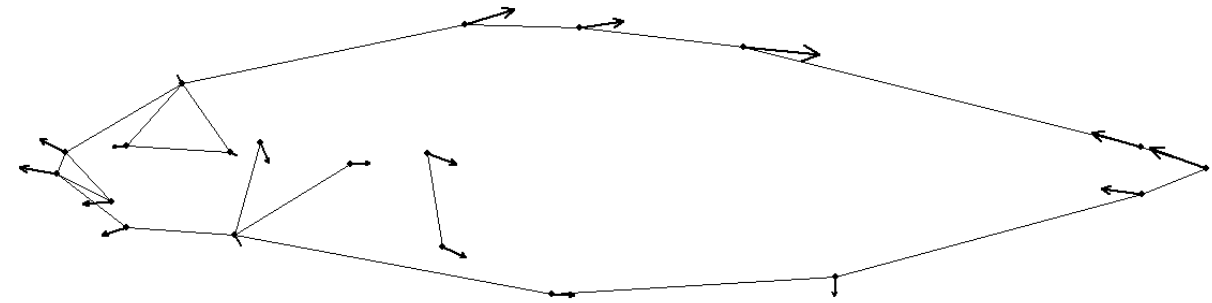

Fig. 2.3 Actual mean shape of sticklebacks. Landmarks represent the shape of the complex habitat fish and vectors represent the shape of the simple habitat fish as a deformation from the complex habitat fish shape.

2.5. Discussion

Exposure to different physical environments, one simple the other complex, in this experiment resulted in the expression of very significant differences in body and head morphologies and spine position.

In a mixed genetic population of three-spine sticklebacks, plastic responses in head shape and spine length have been shown for this species exposed to different diets and predators (Day *et al.*, 1994; Day & McPhail, 1996; Kristjansson *et al.*, 2002; Bell & Sih, 2007; Scotti & Foster, 2007; Wund *et al.*, 2008). Also, the different response of sticklebacks to the ecological surrounding of different habitats was shown by Kristjansson (2005). He found that marine three-spined sticklebacks can change their morphology and armour characteristics extremely quickly when they are acclimatised in freshwater ponds

and that phenotypic changes in this species can occur extremely quickly within one year. However, it was not clear which of all the physical characteristics of the freshwater habitat had more effect on the phenotypic change in the marine sticklebacks.

The phenotypic differences expressed here are apparently well suited to the respective habitats to which each group was exposed. The fish from the complex habitat, which contained rocks and macrophytes, had a shorter distance between the middle and the third spine, whereas in fish from the simple habitat, the distance between second and third spine is increased. The complex habitat fish had a streamlined body, a trait that is likely to facilitate swimming through the rocks and plants easily. Their head shape is also apparently well suited to this habitat, because its small size may aid foraging for prey items in the interstitial spaces between rocks.

Phenotypic plasticity is thought to be important in generating phenotypic diversity observed in nature in many species. It is likely to play a key role in evolution by governing or modifying developmental pathways to produce novel phenotypic traits upon which selection can act (West-Eberhard, 2003a; Fordyce, 2006). The majority of studies examining the expression of phenotypically plastic characters have focused on the role of diet (Adams *et al.*, 1998; Alexander & Adams, 2000; Baker & Foster, 2002; Andersson *et al.*, 2005; Fukumori *et al.*, 2008; Michaud *et al.*, 2008) some studies have shown fitness gains of plastically derived variation in phenotype expression (Jonsson & Jonsson, 2001; Reuter *et al.*, 2008; Witte *et al.*, 2008).

Here it is shown that the physical environment, specifically the complexity of the substratum, can also modulate the expression of traits through phenotypic plasticity during ontogeny and that it is highly likely that the alternative phenotypes expressed are likely to have effects on fitness through their function on foraging ability.

CHAPTER 3. FORAGING SPECIALISM IS PROMOTED BY DISCRETE PREY TYPES AND VARIATION IN TROPHIC PHENOTYPE IN ARCTIC CHARR

*** Note: This chapter will be submitted as a manuscript to Hydrobiologia.**

3.1. Introduction

Dietary specialisations amongst individuals of the same species are relatively common (Lu & Bernatchez, 1999; Maerz *et al.*, 2006; Stuart *et al.*, 2006; Michaud *et al.*, 2008; Woo *et al.*, 2008). In some species, foraging specialisations are extreme and discrete taking the form of discontinuous phenotypes (trophic polymorphisms *sensu* Skúlason & Smith, 1995) with a functional significance for foraging, prey detection, capture or handling (Smith & Skúlason, 1996; Adams & Huntingford, 2002b; Schmidt *et al.*, 2006; Januszkiewicz & Robinson, 2007; Malaquias *et al.*, 2009).

In freshwater fish, multiple examples of sympatric trophic alternative phenotypes have been described (Baumgartner, 1992; Reilly *et al.*, 1992; Larson & Mcintire, 1993; Snorrason *et al.*, 1994; Kristjansson *et al.*, 2002; Yonekura *et al.*, 2002; Swanson *et al.*, 2007; Uchii *et al.*, 2007). Within the three-spined stickleback *Gasterosteus aculeatus* L. many populations have alternative phenotypes specialising in benthic or limnetic foraging that coexist in the same lake. The limnetic form is better adapted to zooplankton consumption having a slender body, long, numerous and densely spaced gillrakers, whereas the more robust benthic form is specialised for feeding on larger food items having less numerous, shorter and widely spaced gillrakers (Foster *et al.*, 1992; McPhail, 1992; Bell & Foster, 1994; Cresko & Baker, 1996; Baker *et al.*, 2005). The limnetic morph of three-spined stickleback is less efficient at feeding on relatively large benthic living organisms and he opposite is true for the benthic phenotype foraging on smaller pelagic living organisms (Schluter, 1993).

Arctic charr *Salvelinus alpinus* L. also frequently exhibit sympatric trophic specialisation. Most frequently this takes the form of a benthic foraging specialist feeding on relatively large macro-invertebrates and a pelagic foraging specialist feeding on planktonic prey (Skúlason *et al.*, 1989; Malmquist *et al.*, 1992; Skúlason *et al.*, 1993; Adams *et al.*, 1998; Alekseyev *et al.*, 2002; Klemetsen *et al.* 2002; Klemetsen *et al.* 2006; Knudsen *et al.* 2006; Knudsen *et al.* 2007; Fraser *et al.*, 2008). In Arctic charr, as in three-

spined sticklebacks, expressed variation in morphology is known to have a functional significance (Smits *et al.*, 1996; Adams & Huntingford, 2002b; Hjelm *et al.*, 2003; West-Eberhard, 2005a; Knudsen *et al.*, 2006; Amundsen *et al.*, 2008). Common to both of these species, and some others for which discrete alternative phenotypes have been reported (e.g. *Coregonus lavaretus*, Ostbye *et al.*, 2005; Kahilainen & Ostbye, 2006), is that they inhabit post-glacial lake systems where the potential foraging resources are typically discrete. The most common prey in these systems are found in different habitat types namely plankton in the limnetic and macrobenthos in littoral zones. They differ significantly in size (Fraser *et al.*, 2008) and differ significantly in the skills needed to forage efficiently on these prey (Schluter, 1993).

There is growing evidence that alternative phenotypes may be important intermediate stages in the route to full speciation (Smith & Skúlason, 1996; Schluter, 2001). In a number of species it is known that diet can shape phenotype expressed in individuals throughout plasticity effects (Queral-Regil & King, 1998; Mittelbach *et al.*, 1999; Starck, 1999; Hegrenes, 2001; Hjelm *et al.*, 2003; Wintzer & Motta, 2005; Olsson *et al.*, 2007; Ruehl & Dewitt, 2007; Ke *et al.*, 2008). It is known that in Arctic charr diet can modulate morphological change in components of the head and mouth which have an important role in foraging efficiency through phenotypic plasticity (Adams *et al.*, 2003b; Adams and Huntingford, 2004). However what is less clear is under what circumstances prey choice and foraging specialisation may develop in individuals in the wild.

3.2. Aims

Here, using Arctic charr, a species which is known to exhibit foraging specialisms and discrete trophic phenotypes (most notably plankton and macroinvertebrate feeding specialisms), we test the degree to which individuals from a monomorphic population exhibit foraging specialisations and the extent to which variations in morphology determine prey choice in individuals exposed to alternative prey. Specifically we test two hypotheses:

a. given a binary choice of prey with different characteristics individuals will specialise in one prey type.

b. individuals will chose prey based on their expressed trophic morphology.

3.3. Methodology

Arctic charr fry supplied by a commercial hatchery (John Eccles Hatcheries), which had been reared in captivity for at least 3 generations but with occasional out crossing to first generation wild fish were used in this study. The stock was originated from two Scottish Arctic charr populations (Loch Luchart and Loch Tay).

Fish (20 months old, 47-83 mm standard length, 75-135mm centroid size) were held in 1m tangential flow, through-flow tanks at temperatures between 16 and 18°C and ambient light, (56ºN). They were fed on standard aquaculture pellet food from first feeding until the experiments started.

3.3.2. *Behavioural trials*

Fish were anaesthetized with benzocaine, marked by Panjet injection in the fins using Alcian Blue and photographed individually on the left side for shape analysis. Twelve specimens were allocated, to each of three 500lt (74 cm x 71 cm x 95 cm) observation tanks, with no substratum or vegetation and a constant flow of water. Fish were initially acclimatised to the tank and deprived of food for 3 days to allow them to recover after marking.

At the beginning of each trial, each observation tank was given two discretely different types of prey. To simulate a pelagic, planktonic prey source, *Artemia sp*. (3-6 mm) embedded in an ice cube of 15x15x4 cm was floated on the surface of the water in the right side of the tank. The ice maintained the *Artemia* in the surface water of the tank and allowed a slow release of the *Artemia* prey as it defrosted. To further prevent *Artemia* dropping to the bottom of the tank, a transparent plastic container was fixed 20 cm below the *Artemia* food source. The container did not obstruct the movements of the fish since it was transparent and the fish were able to observe the *Artemia* easily. To simulate a typical benthic prey, chironomid larvae (8-13 mm) were inserted into agar contained in a Petri dish which was set on the bottom at the left side of the tank. The agar prevented the prey dispersing in the water current. Prey items were available throughout the observation trials in order to prevent competition between fish due to the lack of food. Observations were made once a day between 9:00 and 13:00hrs for 5 continuous days. A focal animal approach was taken where each individual fish was observed for 3 minutes. During each

observation period, the type of prey chosen, number of prey swallowed and any aggression events (nip, chase and attack, see Adams *et al*., 1995) were recorded.

3.3.3. *Morphological analysis*

Homologous landmarks, were identified and placed on photographs of the experimental animals photographed in lateral view, using the software TPSdig2 (Rohlf, 2006; Fig. 3.1). Landmark configurations for each specimen were aligned, translated, rotated and scaled to a unit centroid size using Generalized Procrustes Analysis superimposition (GPA, Rohlf and Slice, 1990) using the consensus configuration of all specimens as the mean shape. Following GPA, new shape variables, Partial Warps (PW), were obtained. In order to explore the overall within-sample form variability, relative warp analysis, equivalent to principal component analysis for morphometric data, was performed on the partial warp scores using the software TPSrelw (Rohlf, 2007).

A specific concept of size was used in the present study, the centroid size (CS), which equals the square root of the summed squared distances of each landmark from the centroid of that landmark configuration. It is measured separately from shape and is uncorrelated to shape in the absence of allometry (Zelditch *et al.*, 2004).

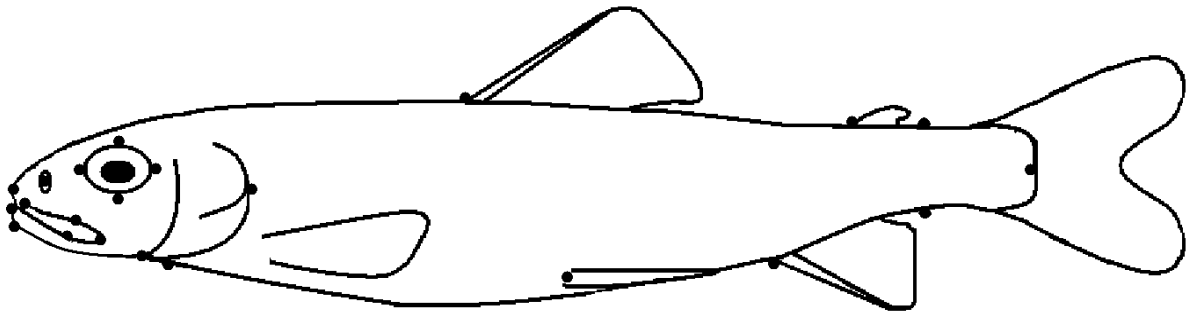

Fig. 3.1 Location of 22 landmarks in juvenile Arctic charr body.

3.3.4. *Data analysis*

ANOVA tests were carried out to compare relative warp scores (morphology) and behavioural (prey consumed and aggression) variables between foraging groups. *Post-hoc* tests with Bonferroni corrections were performed for all shape and size variables. Simple regressions were used to describe relationships among behavioural and morphological variables.

3.4. Results

3.4.2. *Behaviour trials*

A significant number of individuals showed a strong (100%) preference for feeding on only one prey type. Of the 72 fish observed, 39 chose to feed only on chironomids and 12 only on *Artemia*.

Chironomid specialists, *Artemia* specialists and those that switched foraging sources (hereafter called foraging generalists) showed significant differences in the mean total number of prey consumed for all fish over all days ($F_{2,71}$=37.8; p=0.0008; Fig. 3.2). Chironomid specialists took the greatest number of prey (significantly more than both *Artemia* specialists and prey generalists, *post hoc* testing p<0.0001). The prey consumption rate of *Artemia* specialists and generalists was not significantly different (p=0.9).

Twenty-one individuals fed on both foraging sources at least once (foraging generalists), this group did not show a difference between the number of prey items consumed from each source (One-Sample t_{20}=-0.61; p=0.54; Fig. 3.3). The rate of expressed aggression also differed between foraging categories ($F_{2,71}$=17.7; p=0.0006). Chironomid specialists were the most aggressive and the only group that exhibited attack behaviour. Also the frequency of chasing events was notably higher than the other two groups (Fig. 3.4). Aggression and feeding were strongly positively correlated (r^2=0.6, $F_{1,71}$=46.02; p=0.00001) across all groups, where the most aggressive fish (chironomids feeders) also obtained more prey.

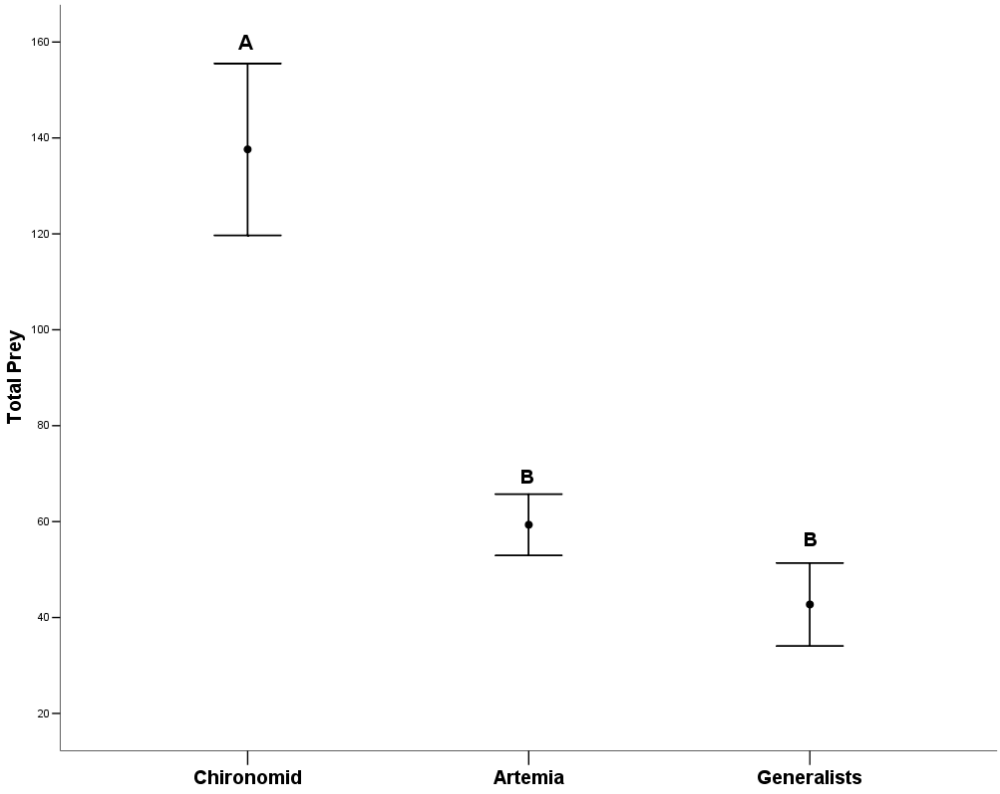

Fig. 3.2 Mean ±SE of total prey consumed by foraging groups. *Post-hoc* testing: similar alphanumeric characters represent not significant differences ($p>0.05$), different alphanumeric characters correspond to significant differences ($p<0.0001$).

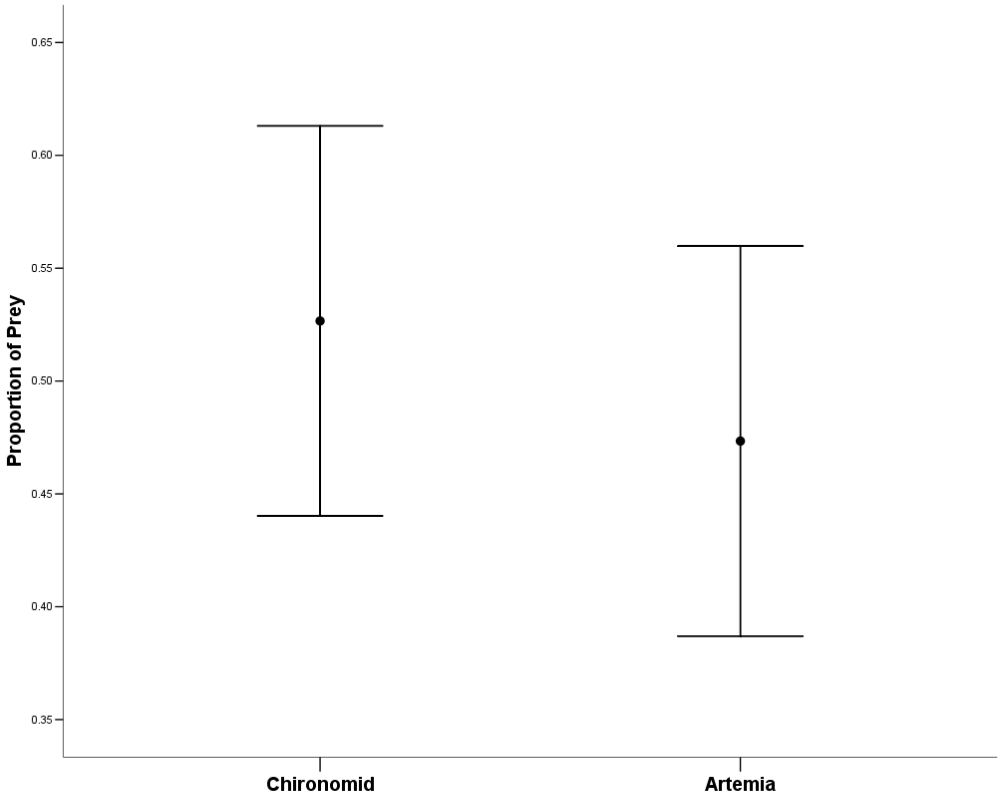

Fig. 3.3 Mean ±SE of the proportion of prey consumed by generalists in five days.

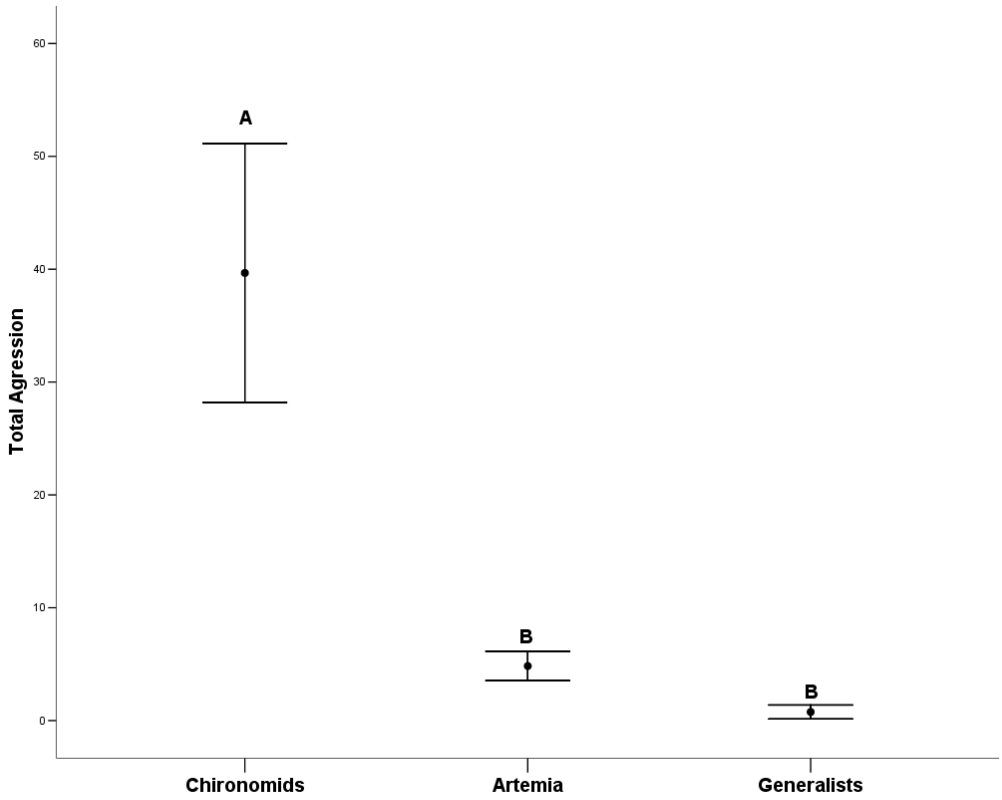

Fig. 3.4 Mean ±SE of total aggressive events by foraging groups in five days. *Post-hoc* testing: similar alphanumeric characters represent not significant differences ($p>0.05$), different alphanumeric characters correspond to significant differences ($p<0.0001$).

3.4.3. Morphology

To reduce the potential effect of size on morphology, only individuals in each of the three foraging groups that overlapped in size were analysed for shape. In total, 42 fish within the range 8.4-10.7 cm of centroid size were used, 12 generalists, 10 *Artemia* and 20 chironomid consumers. A discriminant analysis showed that 88% of individuals assigned to the foraging groups were correctly classified, 16 of 20 (80%) chironomid specialists, 10 of 10 (100%) *Artemia* specialists and 11 of 12 (92%) generalists.

MANOVA analysis run for all relative warps showed that there were significant differences between feeding behaviour groups in relative warp scores (Wilk's $\Lambda = 0.27$, $F_{2,39}= 11.5$; $p=0.001$). Relative warp analysis resulted in three main components that together represent 57.2 % of the total shape variation (see Table 3.1). *Post-hoc* testing showed that generalists had significantly higher RW1 scores than both *Artemia* specialists

(p=0.001) and chironomids specialists (p=0.003), however the latter two were not different from each other (p=0.97).

Table 3.1 General Lineal Model comparing relative warps scores between all foraging groups.

RW	% Variance explained	F	Std. Error	p
1	24.3	9.04	0.0099	0.0006
2	17.7	11.8	0.0080	0.0001
3	15.2	4.5	0.0084	0.018

Fish with positive scores for RW1 showed a reduced head, shorter maxillary bone a smaller eye, also a ventral expansion is perceptible, the posterior section of the body and the head are relatively upturned in contrast with the fish with negative relative warp scores (Fig. 3.5).

For RW2 *post-hoc* testing showed that generalists had significantly higher scores than chironomid specialists (p=0.0001) and higher scores than *Artemia* specialists but not significantly (p=0.4). Meanwhile a significant difference was found between *Artemia* and chironomid feeders (p=0.02). In the second relative warp, fish with positive scores have a more pronounced deeper body in the posterior ventral area, the distance from the anal fin to the end of the caudal peduncle is longer, the head is pointed upwards and the tip of the snout is blunt. In contrast, fish with negative scores are dorsally curved, they present an anterior elongation of the maxillary bone, the snout is slightly sharp and the end of the caudal peduncle is turned down (Fig. 3.6).

Post-hoc testing of RW3 also showed that scores were significantly lower for *Artemia* specialists compared with chironomid specialists (p=0.03) and generalists (p=0.04). Chironomid specialists were distributed in the positive extreme of this component as well as generalists therefore not significant differences were found between these two groups (p=0.9).

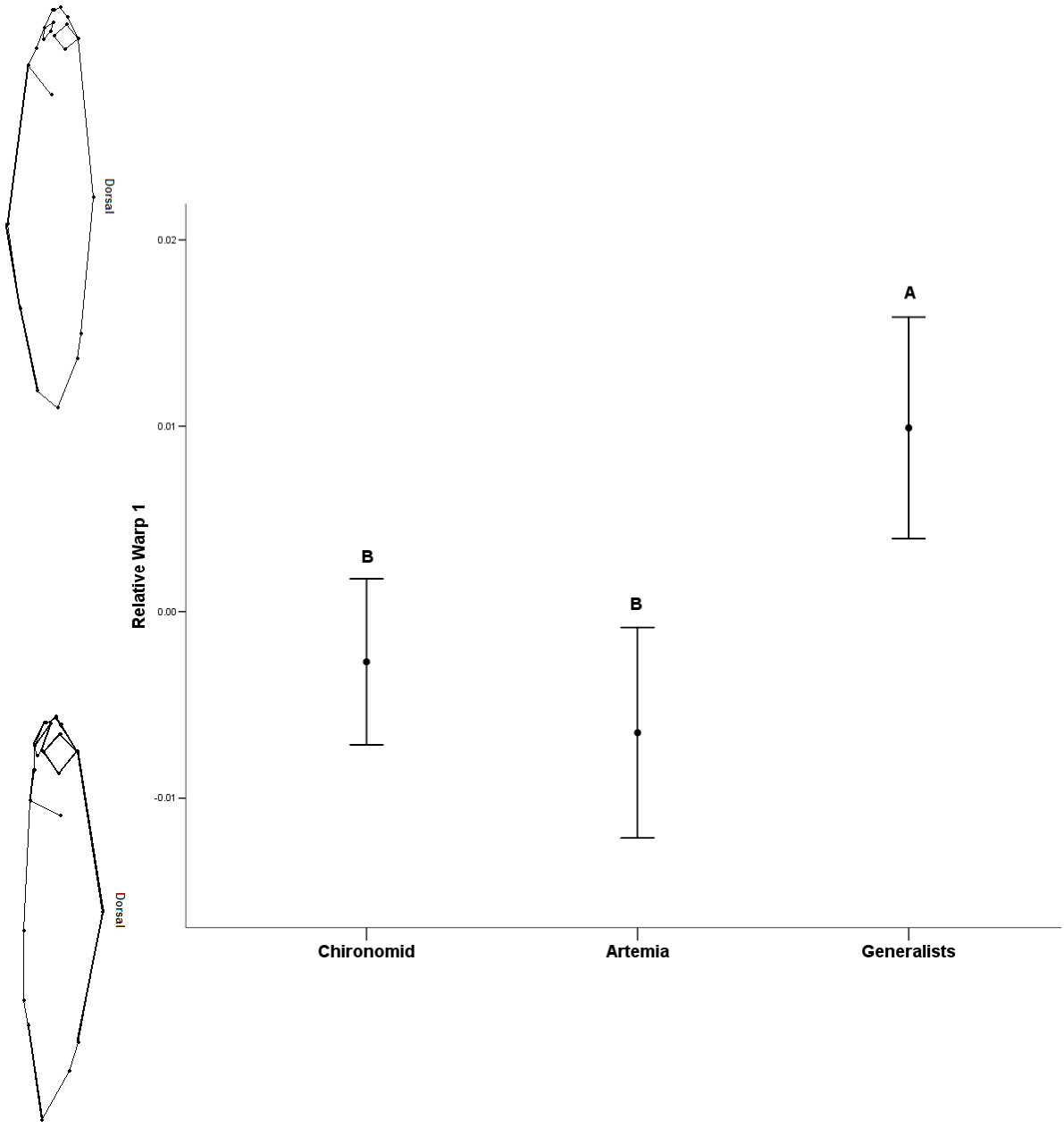

Fig. 3.5 Mean±SE of Relative Warp 1 scores for each foraging group. *Post-hoc* testing: similar alphanumeric characters represent no significant differences ($p>0.05$), different alphanumeric characters correspond to significant differences ($p<0.0001$). On the left side of the plot the upper spline represents the shape of the individuals with positive scores and the lower spline represents the shape of the individuals with negative scores.

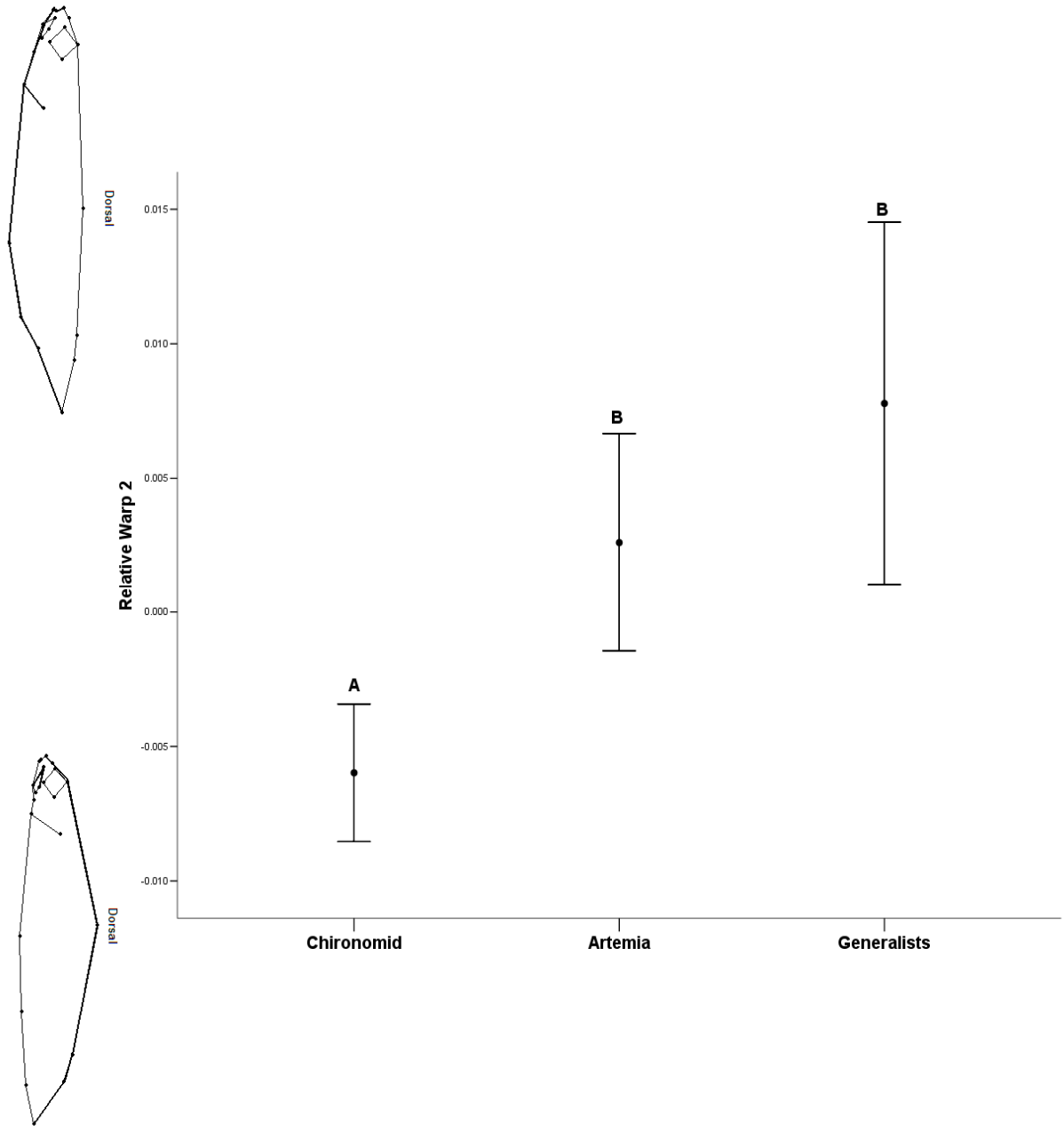

Fig 3.6 Mean±SE of Relative Warp 2 scores for each foraging group. *Post-hoc* testing: similar alphanumeric characters represent no significant differences ($p>0.05$), different alphanumeric characters correspond to significant differences ($p<0.0001$). On the left side of the plot the upper spline represents the shape of the individuals with positive scores and the lower spline represents the shape of the individuals with negative scores.

Fish with negative scores in RW3 exhibit a more slender and fusiform body, larger eye, more elongated anterior part of maxillary bone, the distance between the end of the jaw and the junction of the operculum with the body is noticeably reduced. Also the tip of

the snout is pointed upwards, see Fig. 3.7. A comparison of the actual shape of the specialised feeding groups is depicted in Fig. 3.8.

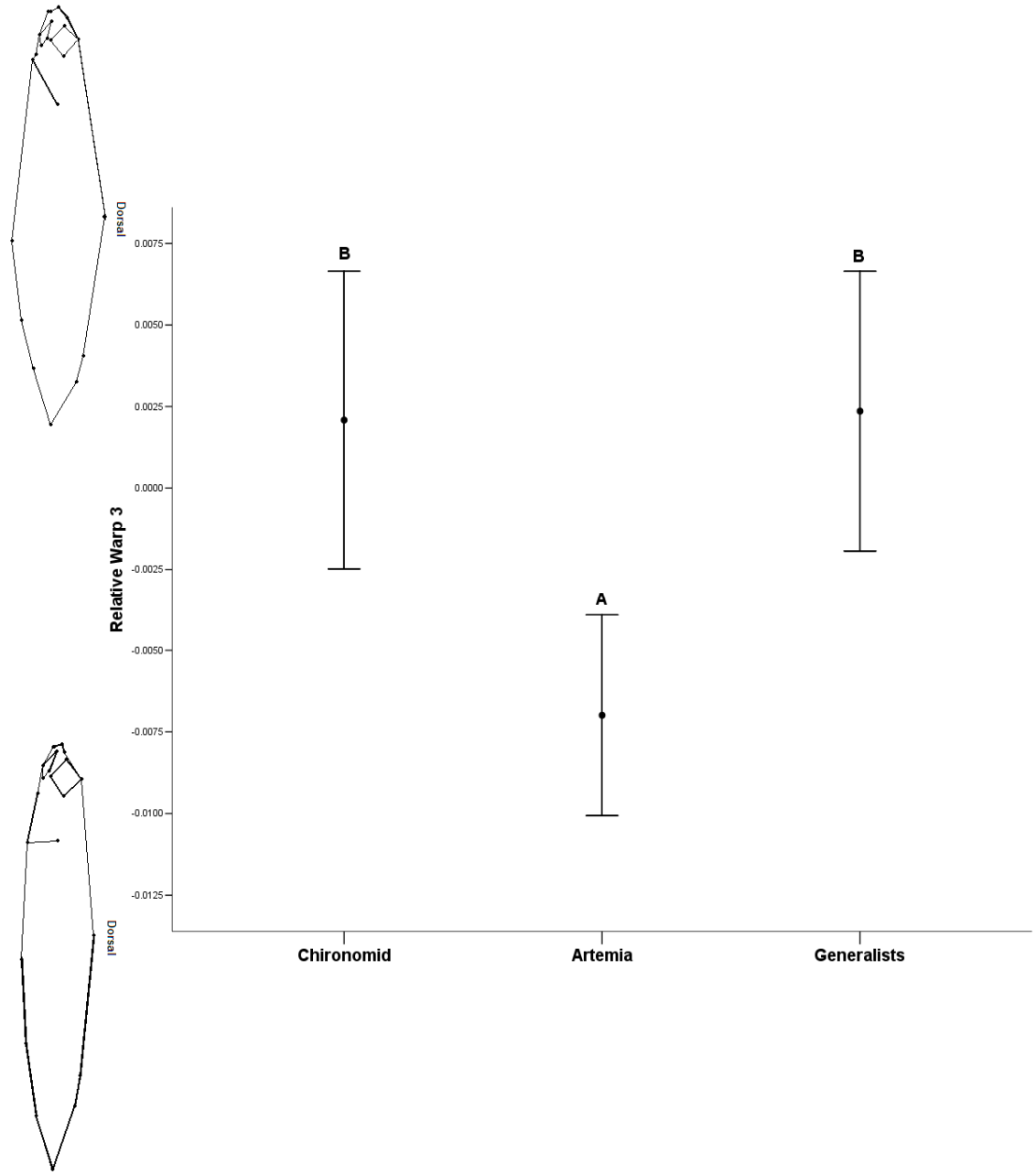

Fig. 3.7 Mean±SE of Relative Warp 3 scores for each foraging group. *Post-hoc* testing: similar alphanumeric characters represent no significant differences ($p>0.05$), different alphanumeric characters correspond to significant differences ($p<0.0001$). On the left side of the plot the upper spline represents the shape of the individuals with positive scores and the lower spline represents the shape of the individuals with negative scores.

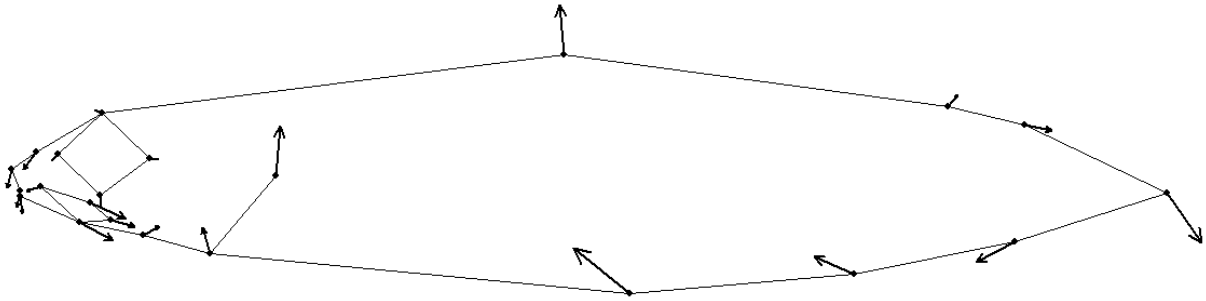

Fig. 3.8 Shape of the Arctic charr individuals. Landmarks indicate the *Artemia* feeders shape and vectors indicate chironomid feeders shape as a deformation from the *Artemia* feeders shape. Landmarks are connected by links to facilitate the visualization shape.

3.5. Discussion

When offered a choice between two prey types, designed to reflect the very discrete prey choices to which fish living in post-glacial lakes are exposed, most individuals (73%) showed complete fidelity to a single foraging source. This strongly supports the suggestion that the benefits of specialising in foraging on a single food source are greater than the costs of switching between food sources. The two prey items offered in this experiment differ very significantly in a number of characteristics, most importantly size, shape and habitat (Werner & Hall, 1974; Kahilainen & Ostbye, 2006; Schmidt *et al.*, 2006; Fraser *et al.*, 2008) and require a different set of behavioural techniques to enable efficient foraging (Maheswaran & Rahmani, 2002; Warburton & Thomson, 2006). They represent the most abundant foraging resources in postglacial lakes (Robinson & Wilson, 1994; Smith & Skúlason, 1996; Robinson & Parsons, 2002; Kahilainen *et al.*, 2007) and the foraging specialisms most frequently described in trophic polymorphic systems (Wainwright *et al.*, 1991; Malmquist *et al.*, 1992; McPhail, 1992; Wimberger, 1994; Adams *et al.*, 1998; Fraser *et al.*, 1998; Swanson *et al.*, 2003; Kahilainen & Ostbye, 2006) and thus may reasonably reflect foraging specialisms choices in the wild for fishes living in postglacial lakes.

In addition, the choice of which foraging specialism to adopt is at least partly based on the trophic morphology of the individual. Here we showed that individuals with chunkier, blunter and bigger mouths, with a more ventral position of the head were more likely to forage on the benthic prey source (chironomids) than on pelagic prey. In contrast, individuals characterised by a slender and fusiform body, bigger eye but slightly small

body size were more likely to be pelagic prey (Artemia) than benthic prey specialists. Overall, Generalists did not feed significantly more on benthic prey than planktonic prey and showed morphological differences from the two specialist groups: shorter maxillary bone, a smaller eye, ventral expansion, with the posterior section of the body and the head relatively upturned.

Because morphology was measured before fish were exposed to the experimental conditions, the phenotypic variation between foraging groups was not the result of exposure to different diets and thus are not the result of a phenotypic plasticity response to diet but the result of a natural, continuous variation in morphological characteristics.

Models that invoke trophic specialisation as a driver for evolutionary divergence (Dieckmann & Doebeli, 1999; see e.g. Skúlason *et al.*, 1999) propose that divergence begins with behavioural changes in prey choice, which are themselves shaped by opportunities to use resources (Skúlason & Smith, 1995). Foraging specialisms may then result in morphological change through diet induced phenotypic plasticity that results in increased foraging efficiency and therefore reinforce the foraging specialism (Robinson & Parsons, 2002; Adams *et al.*, 2003b; Michaud *et al.*, 2008). Here we have shown that when exposed to a binary prey choice where prey types differ significantly in a number of ecological characteristics that affect their accessibility as prey, individuals predominantly specialise in one prey type and that this initial foraging specialism is at least partly determined by small inter-individual variations in morphology which has been considered important since morphological differences related with strong segregation in behaviour, habitat and food have been found between sympatric phenotypes of lacustrine Arctic charr (Skúlason *et al*. 1983; Klemetsen *et al.*, 2002; Klemetsen *et al*. 2006). Although the results presented here showed that chironomid feeders were more aggressive, dominance over *Artemia* specialists is unlikely to represent an explanation for the diet preferences because the *Artemia* specialists did not tried to feed in the chironomid territory. It is now clearly established that long term specialisation on diets that are discretely different in nature can and does result in significant morphological divergence through ontogenetic plasticity effects in this species (Noor, 1999; Adams *et al.*, 2003b). A logical consequence of this is that small subtle variations in morphology in conjunction with foraging fidelity and plasticity could result in discrete alternative phenotypes in sites where distinct and discrete prey types are present. Recently de-glaciated freshwater lakes provide one common ecosystem type where these conditions exist.

CHAPTER 4. ASSORTATIVE MATING CHOICES BASED ON EXPRESSED PLASTIC PHENOTYPIC TRAITS IN THREE-SPINED STICKLEBACK

***Note: This chapter will be submitted as a manuscript to the "Proceedings of the Royal Society B"**

4.1. Introduction

There is a growing understanding that phenotypic variation arising from ontogenetic responses to the environment (phenotypic plasticity; West-Eberhard, 1989), has the potential to provide significant phenotypic novelty upon which selection may act. Where this results from the expression of one or more alternative phenotypic traits, selection can potentially act semi-independently on multiple phenotypic modes (West-Eberhard, 1989; 2003a). Alternative phenotypes which are discrete in nature and associated with an ecological function such as foraging (trophic polymorphism *sensu* Skúlason & Smith, 1995; Smith & Skúlason, 1996) have been strongly implicated in sympatric speciation events (Maynard-Smith, 1966; Dieckmann & Doebeli, 1999; Schluter, 2001).

The role of the environment, and particularly the ecological environment, to which organisms are exposed in modulating the expression of phenotype has been described for a number of species which have plastic traits (Meyer, 1987; Wimberger, 1992; Day & McPhail, 1996; Mittelbach *et al.*, 1999; Adams *et al.*, 2003a; Alexander & Adams, 2004). However, expression of alternative phenotypes does not result in evolutionary change without a mechanism resulting in gene pool segregation (Skúlason *et al.*, 1996; Schluter, 2003; West-Eberhard, 2003a). This evolutionary step has proven difficult to both conceptualise and to demonstrate empirically (Dieckmann & Doebeli, 1999) but is critical to the process of ecological speciation (West-Eberhard, 1989; Schluter, 2001; West-Eberhard, 2003a). Here we test one route through which plastic phenotypic novelty could potentially result in evolutionary change; the selection of plastic traits by females during reproduction.

Assortative mating in three-spined sticklebacks, *Gasterosteus aculeatus*, is well known but the actual criteria used in mate choice are not fully understood. There is evidence that males display preferences for different sizes of females (Albert & Schluter,

2004) and that females choose to mate assortatively by size (Hatfield & Schluter, 1996; Nagel & Schluter, 1998; Rundle & Schluter, 1998; Albert, 2005).

It is known that female choice is also based on factors like nuptial colour pattern (Scott, 2004), nest site and structure (Blais *et al.*, 2004), courtship behaviour (Ólafsdóttir *et al.*, 2006), habitat choice (Vamosi & Schluter, 1999), and symmetry of spines (Mazzi *et al.*, 2003).

4.2. Aims

Here we test the effect of diet on the development of body shape variation in sticklebacks and the consequent effect on mate selection. Specifically, first we test whether diet itself acts as the proximate mechanism to induce changes in body shape and trophic morphology in three-spined sticklebacks. Secondly, we test if body morphology is a proximate selection cue for assortative mating.

4.3. Methodology

4.3.2. *Fish sampling and holding conditions*

Fry of freshwater three-spined sticklebacks were collected, using a dip net, from a pond adjacent to the Endrick River, Stirlingshire, Scotland (56°3'N; 004°21'W), in July 2006. In total 240 juveniles (5-9mm TL) were caught and transported within 1 h to rearing facilities at the Scottish Centre for Ecology and the Natural Environment (SCENE), Glasgow University, Loch Lomond. Fish were assigned randomly, in groups of forty, to 6 21-litre holding aquaria prepared with rocky substratum and continuous water flow. Water temperature was held at ambient Loch Lomond. The specimens were raised in the laboratory until reaching sexual maturity (11 months).

4.3.3. *Diet treatments*

Sticklebacks were split into two diet treatments (3 aquaria per treatment) and fed two times daily to satiation for 11 months. The two treatments were intended to induce an effect of morphological plasticity (Day & McPhail, 1996). To simulate a pelagic prey diet,

one treatment group was fed only frozen *Daphnia sp.* This was provided to the fish in a hanging bag made of plastic mesh. The second treatment was designed to simulate a benthic prey diet and consisted of frozen chironomid larvae fed on the bottom of the tanks.

4.3.4. *Morphological analysis*

After 10 months of exposure to one of the two diet treatments, each stickleback was anaesthetised with benzocaine and photographed on its left side with a Canon EOS digital 350D camera (8.0 megapixels) which was fixed to a camera stand. Each fish used in mate choice experiments was re-photographed at 11 months when the fish were sexually mature immediately following mate choice experiments.

The overall body size and shape were quantified using landmark configurations. Twenty landmarks were set on the digital images (see Fig. 2.1) using the computer software tpsDig2.1 (Rohlf, 2006a). Thereafter the TWOGroup6 program from the IMP series (Sheets, 2003) was use to performed a Goodall's F-test to test for mean shape differences between the two diet treatments.

To summarise the morphological differences the Procrustes distance between each pair of individuals used in mate choice trials, was computed with the tpsSmall program (Rohlf, 2003). This metric defines an inter-object shape distance, obtained after the landmarks superimposition, defined as the square root of the sum of the square distances between two centred (superimposed centroids), normalised (centroid size=1) and optimally rotated configurations of landmarks (Rohlf *et al.*, 1996; Antani *et al.*, 2004). A Relative Warp Analysis (similar to Principal Component Analysis) using TPSRelw software (Rohlf, 2007) was used to quantify shape variation and similarity between females and males.

4.3.5. *Mate choice trials*

By June 2007, males had developed nuptial coloration (red throat, and/or blue iris colour and bluish body sheen) and some of the females were gravid, therefore they were judged ready to use in mate choice trials. Due to a high rate of mortality during the rearing period and the slow growth of the fish fed on *Daphnia sp.*, only sixty-four threespined

sticklebacks were used, 28 females (21 chironomid diet, 7 *Daphnia sp.* diet) and 36 males (21 chironomid diet, 15 *Daphnia sp.* diet), for the mate choice trials.

The mating trials were carried out using a visual contact experiment similar to that of Seehausen (1997). A single gravid female was placed alone in an aquarium with a view of two males, held in separate adjacent aquariums that did not have visual contact with each other. Choice experiments were conducted using only size-matched males that were unfamiliar to the female (i.e. from a different rearing tank). The mean difference in size between males in a pair for all trials was: $\bar{x}$=0.039cm± S.E=0.023.

Tanks of 16x29x19 cm were used for the observation experiments; the two males were introduced in the same tank but were separated from each other with an opaque plastic division, splitting the tank into two sections of equal size. Prior to introducing the fish, tanks were filled with lake water and three walls of each aquarium were covered with black plastic to avoid distracting the fish with movements outside the tanks. The only items in the tank were a thermometer and a heater. The female and males were acclimatised to experimental conditions of 18ºC, fed *at libitum* and left for 12h (overnight) in the observation tank on the day preceding testing to reduce possible exploratory behaviour during the test. The tanks were separated with a dark plastic divider to prevent the female seeing any males before the experiments started. Combinations of two size-matched males (comprising chironomid-chironomid diet treatment, chironomid-*Daphnia sp.* diet treatment or *Daphnia sp.* - *Daphnia sp.* diet treatment) were tested separately. At the start of the trial the female was enclosed in a bottomless plastic container in a central section of the tank (Fig 4.1).

The trial began when the dark plastic divider was removed, and the female released from the plastic container. Fish usually started to interact visually almost immediately. Each trial lasted for 5 minutes, during this period the time the female spent in the side of the tank corresponding to each of the two males was recorded. Male consorting time was recorded only when the female occupied the two quarters of the tank nearest to the males (hatched area in Fig. 4.1). To be sure that female choice was based on male presence instead of her preference for one of the sides in the tank three replicates of each pairing trial were conducted swapping the male position each time. The male chosen by the female was defined as the male with which the female spent more time (Kraak & Bakker, 1998). Males and females were used maximally in four trials in different days; males were re-used

in fresh combinations so that the female was never given the same male to choose. All individuals involved in male choice experiments were subsequently analysed for morphology.

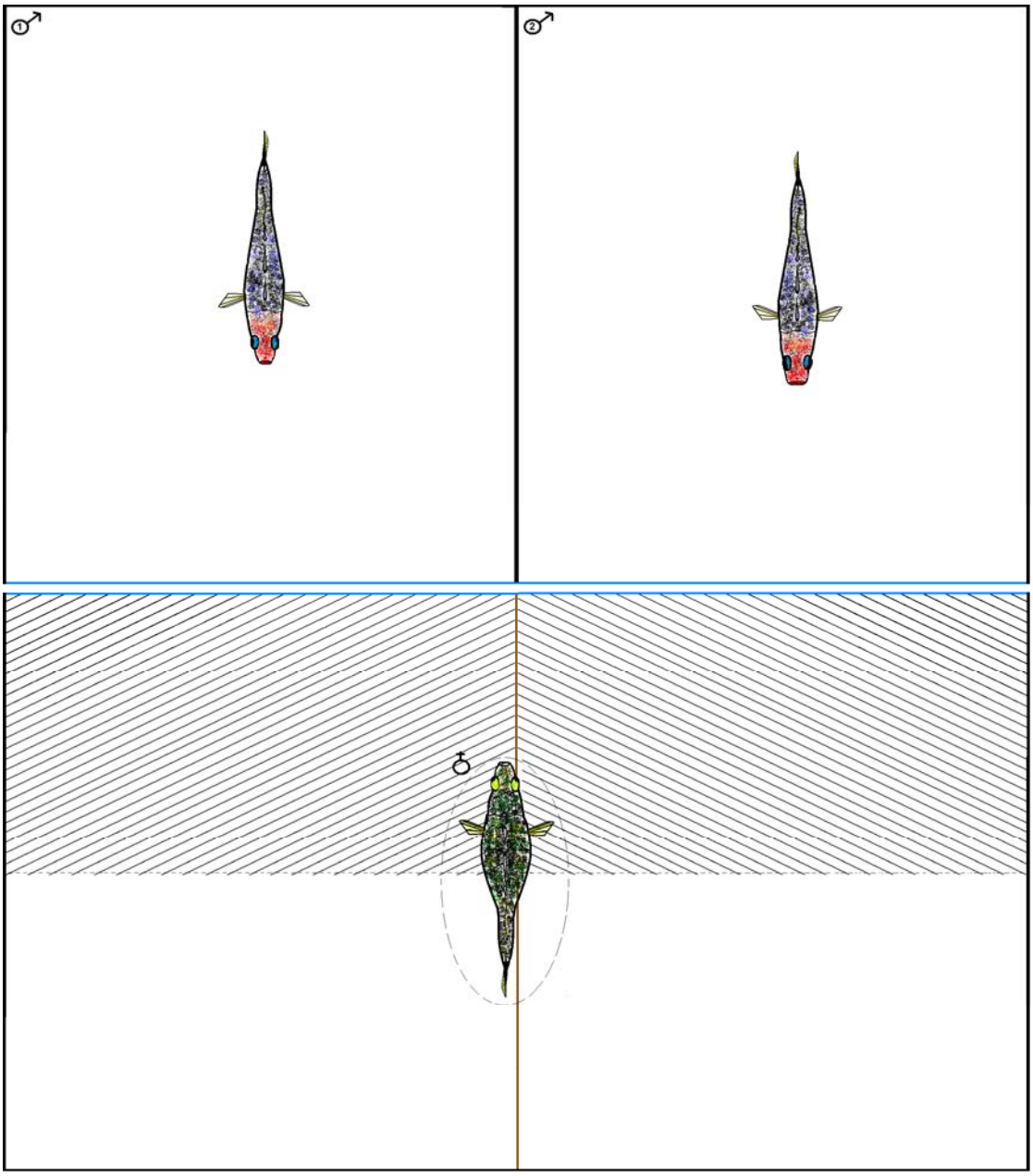

Fig. 4.1 Diagram of the experiment-tank set-up. Long-dash circle surrounding the female represent the bottomless plastic container at the beginning of each trial. Hatched area indicates the two quarters of the tank where the time was recorded, the short-dash line represent the limit.

4.3.6. *Statistical analysis*

Female preference was calculated as the proportion of time spent with each male. Preference scores were arcsine, transformed to normalise data. Binomial test, chi-squared of independence test and *t*-test were performed as appropriate in order to compare the behavioural response of females. A female was deemed to have "chosen" a male if she spent at least 60% of the total time of the trial with the male. ANOVA tests were used to compare morphological differences.

4.4. Results

4.4.2. *Effect of the diet on Morphology*

Sticklebacks exposed to different diet treatments for 10 months showed a significant divergence in body shape between treatments (Goodall's $F_{36, 2232} = 3.6705$; $P< 0.001$). To visualise shape differences among chironomid and *Daphnia sp.* diet groups, a spline deformation with vectors displacements was generated (Fig. 4.2). The first three relative warps, together explained 63% of the total shape variation in the data from sexually mature individuals (Table 4.1), RW1 scores mostly described differences in shape among sexes and was not considered further here. Thus, only RW2 scores were chosen to explain the effect of the diet treatment on morphological variation, because it showed significant differences among chironomid fed and *Daphnia sp.* fed individuals ($F_{1,62}=22.2$; $p< 0.0001$). Fish fed on *Daphnia sp.* had larger head, longer maxillary bone, larger eye and slimmer body (Fig. 4.3).

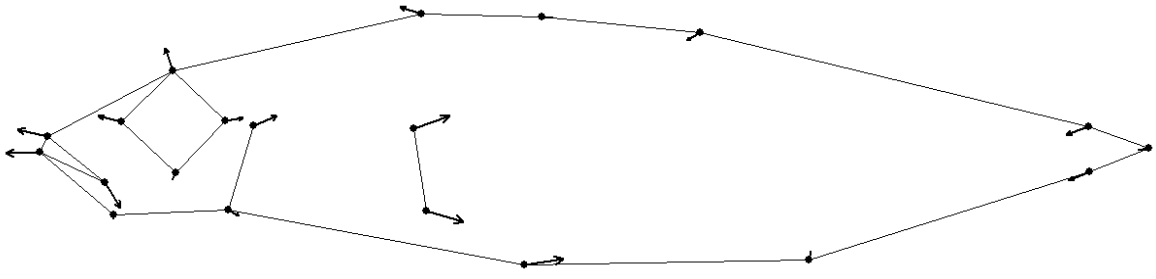

Fig. 4.2 Landmarks configurations for three-spine sticklebacks. The landmarks are connected by links to aid visualisation of fish shape. Vectors indicating displacements represent the *Daphnia sp.* diet treatment fish shape showed as a deformation from the chironomid diet shape.

Table 4.1 General Lineal Model results of the effect of diet treatment, sex and their interactions for each of the first three relative warps of the shape analysis. Significant values of **p** are indicated with a star.

Relative Warps	**%** Variance Explained		df	Mean Square	F	p
1	40	**Sex**	1	0.012	54.6	0.00*
		Diet	1	0.000	1.3	0.2
		Sex * Diet	1	0.001	5.05	0.03*
2	13	**Sex**	1	0.001	3.1	0.08
		Diet	1	0.005	22.2	0.001*
		Sex * Diet	1	0.001	2.9	0.1
3	10	**Sex**	1	0.003	12.9	0.001*
		Diet	1	0.002	8.8	0.004*
		Sex * Diet	1	0.001	4.9	0.03*

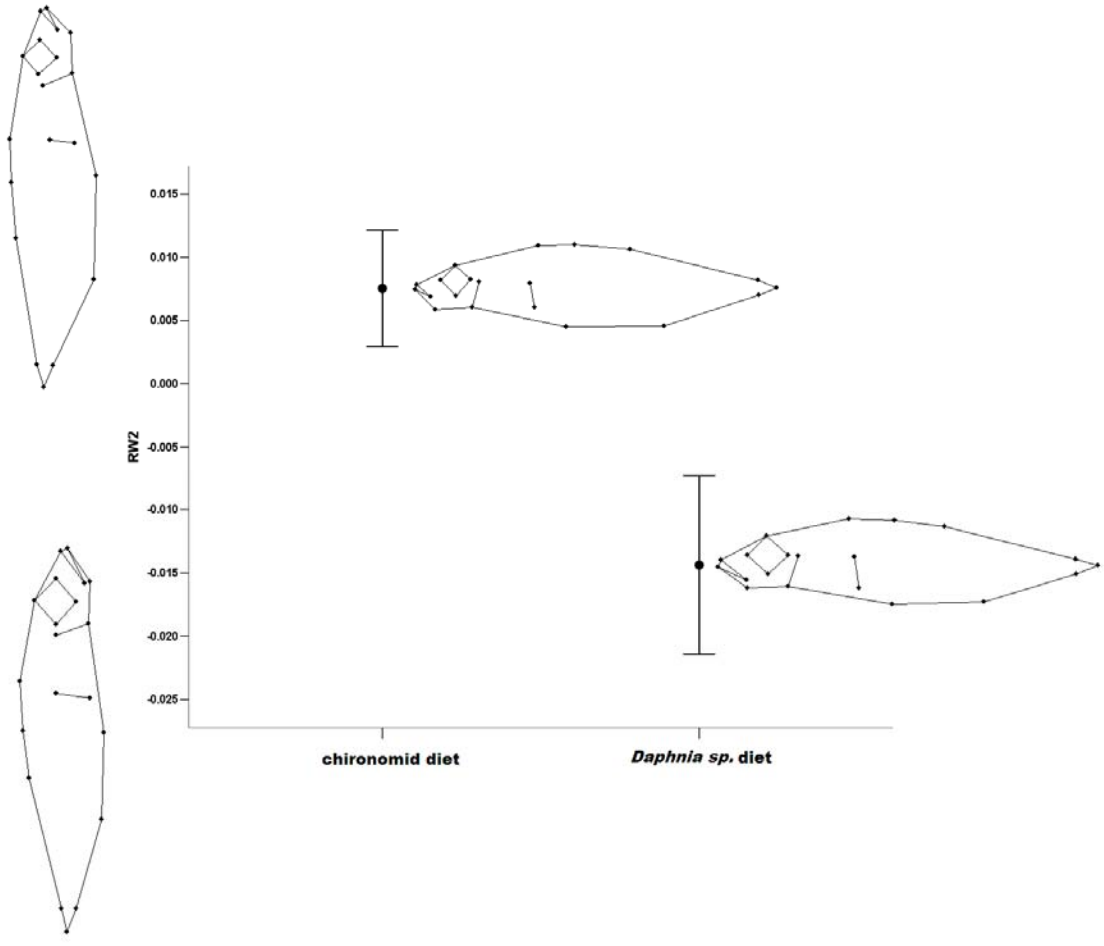

Fig. 4.3 The RW2 scores mean and standard error of chironomid diet treatment and *Daphnia sp.* diet treatment individuals.

4.4.3. *Mate choice trials on the basis of diet treatment*

There was evidence of assortative mating on the basis of diet treatment in these trials (all females both diets χ^2_1=29.1; p<0.0001). Of 44 trials run using *Daphnia sp.* diet females, 91 % of them chose *Daphnia sp.* diet males over chironomid diet males. Of the 68 trials using chironomid diet females, 60% of females chose chironomid diet males over *Daphnia sp.* diet males.

4.4.4. *Mate choice trials on the basis of RW2 scores*

The RW2 score of each fish was used to quantify the position of each fish on a continuum ranging from highly "pelagic like" (low RW2 score) to highly "benthic like" (high RW2 score). Also to highlight the differences between extreme scores along the RW2 continuum five females (4 chironomid fed and 1 *Daphnia sp.* fed), with scores near to zero (0±0.003) were eliminated from the analysis. Then, morphological distances among females and chosen and rejected males were calculated based on RW2 scores.

Although male pairs were matched in size to avoid known female size preference, small body size discrepancies between pairs remained. Therefore, female choice was tested for any residual effect of body size on mate choice. Females did not have preference for larger or smaller body size amongst (almost size matched) male pairs, (Table 4.2). The mean size difference between female and chosen male was $\bar{x}$=0.3cm+ S.E=0.04, between female and rejected male $\bar{x}$=0.4cm+ S.E=0.04 and between males $\bar{x}$=0.04cm+ S.E=0.02 (see Table 4.2).

Females chose the male that was more similar (closer in RW2 score to her morphology) in 75% of trials ($F_{1,86}$=5.8, p=0.02). Also, there was a statistically significant correlation between the relative warp scores of the female and the chosen male (r=0.2, $F_{1,69}$=4.4, p=0.04) (Fig. 4.4), females with positive scores preferred males with positive scores likewise females with negative scores preferred males with negative scores. This suggests that females had a positive preference for males with morphology generally more similar to hers.

Table 4.2 Binomial Tests results of comparisons among traits within the morphometric, diet and size alternative characteristics of males. Data are based on RW2 scores.

Variable	Male trait	N	Observed Prop.	Test Prop.	p
Morphometric Distance to female	Similar	45	0.63	0.5	0.03
	Dissimilar	26	0.37		
Diet	Same Diet	47	0.66	0.5	0.009
	Different Diet	24	0.34		
Size	Small	37	0.52	0.5	0.8
	Large	34	0.48		

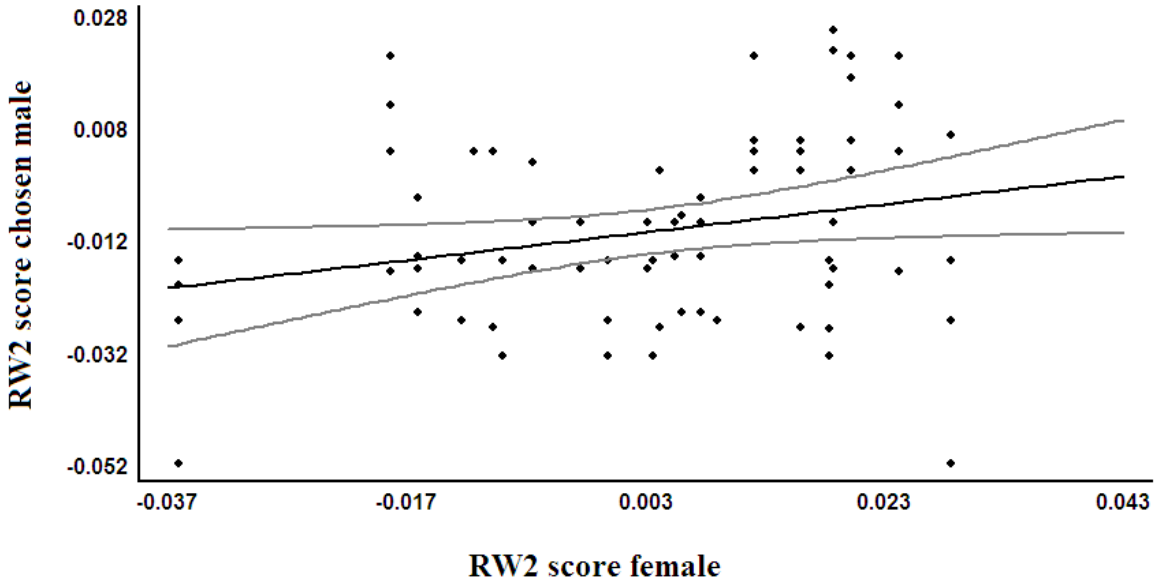

Fig. 4.4 Plot of the RW2 scores for both females and males showing assortative preference of females for morphologically similar males.

The strength of the preference for an individual male in the pair was tested by comparing the proportion of time that the female spent with the chosen male in defined similar and dissimilar male pairs. More benthic-like females did not spent significantly more time with chosen males when the males were dissimilar compared with when they

were similar ($F_{1,87}$=0.054; p=0.82), but more pelagic females spent more time with more pelagic males than with more benthic males ($F_{1,53}$=8.06; p=0.006) (Fig. 4.5).

The position of males and females along the RW2 continuum was analysed to determine if the females were choosing males of more extreme morphology. Forty-one out of seventy-one chosen males showed a more extreme morphology. There was no significant preference for males with more extreme morphologies than that of the female making the choice (Binomial test, N=71, p=0.23).

However, a strong relationship between similarity and extreme morphology was found. Chi-square test suggested that if the male is "similar" and "more-extreme" is more likely to be chosen than a "dissimilar" and "less-extreme" male (χ^2_1=35.89, p<0.0001)

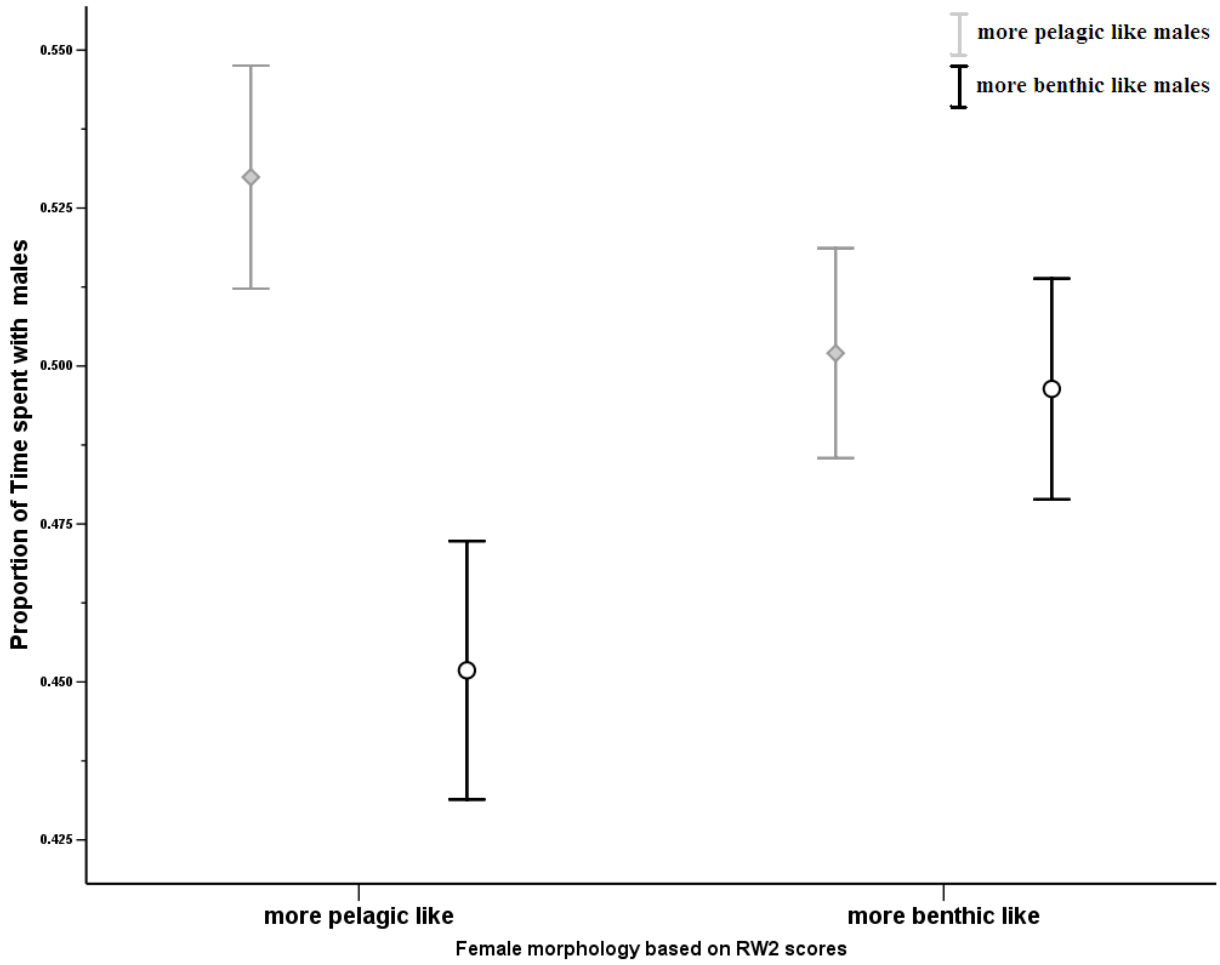

Fig. 4.5 The Mean± standard error of the proportion of time that females spent with males. Females and males are grouped based on their morphology defined by their RW2 scores.

4.5. Discussion

Diet had a significant effect on the morphology during development of the three-spined sticklebacks in this study. Here the induced changes in the morphology of sticklebacks mainly comprise changes in the shape of the head. The results presented here strengthen the evidence that diet plays a key role to the development of morphological divergence and highlight the effect of morphological plasticity in this species (Day & McPhail, 1996; Hegrenes, 2001). The data here are consistent with the common description of sympatric morphologies of sticklebacks from natural populations (Schluter & McPhail, 1992; Foster *et al.*, 1992; McPhail, 1992). Fish fed on *Daphnia sp.* developed a very similar morphology to limnetic fish and distinct to benthic fish; longer snout, slender body and bigger eye (see page 57; McPhail, 1992). The characteristic trophic morphology that arose as an effect of the diet shows that non-genetic factors can cause phenotypic divergence through phenotypic plasticity (West-Eberhard, 2003b).

During their developmental period, in rearing conditions, females coexisted with males that shared the same habitat and used the same resources as them. Because all the individuals were removed from their natural habitat very early stage in their development, sexual imprinting based on appearance of their parents (Albert, 2005) or other parental imprinting are highly unlikely to have an effect on their sexual preferences (Todd & Miller, 1993) in this study.

The three-spined sticklebacks from the River Endrick have been shown to be highly efficient in their ability to learn spatial tasks, orientation and displays (Girvan & Braithwaite, 1998; Girvan & Braithwaite, 2000). Thus it is reasonable to suppose that they have high visual acuity ability (Mazzi *et al.*, 2003) enabling them to identify the shape of other fish.

Results of the choice experiment here show clear evidence of assortative mating on the basis of body shape. Females chose to mate with males that are more benthic like or limnetic like depending on their own morphology. Thus, limnetic like females were more likely to chose limnetic like males and benthic like females were more likely to choose benthic like males, although the strength of this latter effect was weaker than the former. An interesting finding of this study is that females chose males closer to their morphology rather that a more extreme morphology to theirs.

Models of evolutionary divergence invoking phenotypic plasticity as one step in the process of divergence assume that phenotypic plasticity can provide phenotypic novelty upon which divergent selecting forces may act (West-Eberhard, 2003b). However, this process can not result in divergent evolutionary change without gene pool segregation. Here we demonstrate one potential sexual selection route through which gene pool segregation may occur. Our results suggest that pre-isolation phenotypic divergences by means of developmental plasticity have a consequence in the mating behaviour in the three-spine sticklebacks, which becomes assortative for plasticity induced morphological traits. These results represent the first experimental work that supports the hypothesis that assortative mating may facilitates speciation because it can cause rapid evolutionary diversification (Dieckman and Doebeli, 1999; Kondrashov and Kondrashov, 1999; Boughman, 2001; Coyne, 2004, Bagnoli and Guardiani, 2008; Bolnick and Fitzpatrick, 2007; Bolnick and Lee, 2008).

THREE-SPINED STICKLEBACKS

Pelagic-like male

Pelagic–like Female

Benthic-like male

Benthic-like female

CHAPTER 5. ECOLOGICAL, MORPHOLOGICAL AND GENETIC EVIDENCE OF ALTERNATIVE EVOLUTIONARY ORIGINS IN ARCTIC CHARR (*SALVELINUS ALPINUS*) FROM TWO ALTERNATIVE-PHENOTYPE SYSTEMS IN SCOTLAND

***Note: This chapter has been submitted as a manuscript to "Evolutionary Ecology" journal.**

5.1. Introduction

The occurrence of two or more discrete phenotypes among individuals within a species, is now widely regarded as one stage on the route to speciation and particularly so if it occurs in sympatry (Schluter & McPhail, 1992). The expression of one or more discrete phenotypes provides multiple, alternative modes upon which selection can act semi-independently and thus has the potential to drive alternative phenotypes towards different evolutionary outcomes (West-Eberhard, 2003a). This effect is particularly evident where alternative phenotypes are expressed in sympatry (Schluter & McPhail, 1992) and where the expressed phenotypes have a strong functional significance (West-Eberhard, 2005a). Thus examination of sympatric alternative phenotypes, amongst traits that have significant ecological importance for the organisms expressing those traits, has the potential to offer unique insights into the selective forces and evolutionary processes shaping change.

The coexistence of alternative forms of freshwater fish, differing in traits that have a role in foraging, are known to be relatively common in post-glacial lake systems throughout the holarctic (Schluter & McPhail, 1992; Wimberger, 1994; Skúlason & Smith, 1995; Smith & Skúlason, 1996). There is now a robust and growing literature that demonstrates the expression of two or more discrete suites of alternative phenotypic traits that correlate with alternative foraging ecology in fishes from a range of evolutionary lineages including three-spined stickleback, *Gasterosteus aculeatus* (Schluter, 1993; Baker *et al.*, 1995; Vamosi & Schluter, 2004), whitefish, *Coregonus lavaretus*, (Bernatchez & Dodson, 1990; Bernatchez *et al.*, 1996; Kahilainen & Ostbye, 2006) and Arctic charr, *Salvelinus alpinus* (Klemetsen *et al.*, 2003a). Amongst Arctic charr, sympatric foraging specialisms, most frequently comprising individuals specialising in preying upon plankton, macro-invertebrate benthos or fish, accompanied by discrete morphological variation in

functionally significant traits (Adams & Huntingford, 2002b), have been described from a number of post-glacial lakes throughout the species' distribution (Snorrason *et al.*, 1994; Adams *et al.*, 1998; Klemetsen *et al.*, 2002; Alekseyev *et al.*, 2002).

Two alternative origins for coexisting phenotypes of intralacustrine fish have been suggested. They can be either originated by intralacustrine divergence of one founder population (sympatry) or by multiple invasions of the forms representing different lineages (Robinson & Wilson, 1994; Pigeon *et al.*, 1997; Alekseyev *et al.*, 2002).

Alternative body-size phenotypes have been described previously from Loch Tay (Scotland) Arctic charr (Adams *et al.*, 2003a). Sexually mature charr showed a bimodal length-frequency and ranged in size from 190mm to 290mm (large-body-size) and 80 to 160 mm (fork-length) (small body-size) with no overlap in body-size. Loch Tay is a component part of the Tay system which drains east to the North Sea (Fig 1). Loch Awe has no freshwater connection with the Tay system and drains west into the North Atlantic (Fig.1). The Arctic charr population in Loch Awe is known to segregate into components that spawn in autumn or in spring (Alexander & Adams, 2000; Kettle-White, 2001).

5.2. Aims

Here these alternative phenotypes within lakes are used to address a series of questions relating to their status and the evolutionary processes that led to their formation. Specifically five hypotheseswere tested: that the phenotypes in each lake 1) represent ecologically distinct units, 2) differ in functionally significant morphological characteristics, 3) exhibit different life history traits, 4) represent genetically distinct units and 5) show similar patterns of evolutionary divergence.

5.3. Materials and Methods

5.3.2. *Study areas and sampling*

Arctic charr were collected from Loch Tay, Perthshire (56°30' N; 004°10' W) and Loch Awe (56° 20' N, 005° 05'W), located in Argyll and Bute, West Central Scotland, Fig. 5.1. Fish in Loch Tay were collected during the spawning season (October) 2006; meanwhile, fish from Loch Awe were caught during the spawning seasons for this

population, from the 8th to 15th November 2006 (autumn) and from 21st to 26th February 2007 (spring). Autumn sampling in Loch Awe was conducted at known spawning sites (56°22'21.1" N, 005°4'24.6"W). Sampling in spring was conducted at a different but known site for spring spawners (56°15'06.3" N, 005°16'24.1" W).

Sampling at all sites was carried out using standard benthic Nordic mono-filament survey gill-nets, comprising 12 panels, ranging in mesh size from 5 to 55mm, knot-to-knot. The nets were set on the bottom of the loch overnight and placed perpendicular to the shore, in possible spawning sites for this species.

Collected specimens were brought to the laboratory within 3 hours; each individual was photographed on the left side, measured (standard length ± 1mm), weighed (Seehausen & van Alphen, 1998) and their sex and maturity status determined. Otoliths were removed for age determination. Samples for genetic analysis were taken from the adipose fin and preserved in 100% ethanol.

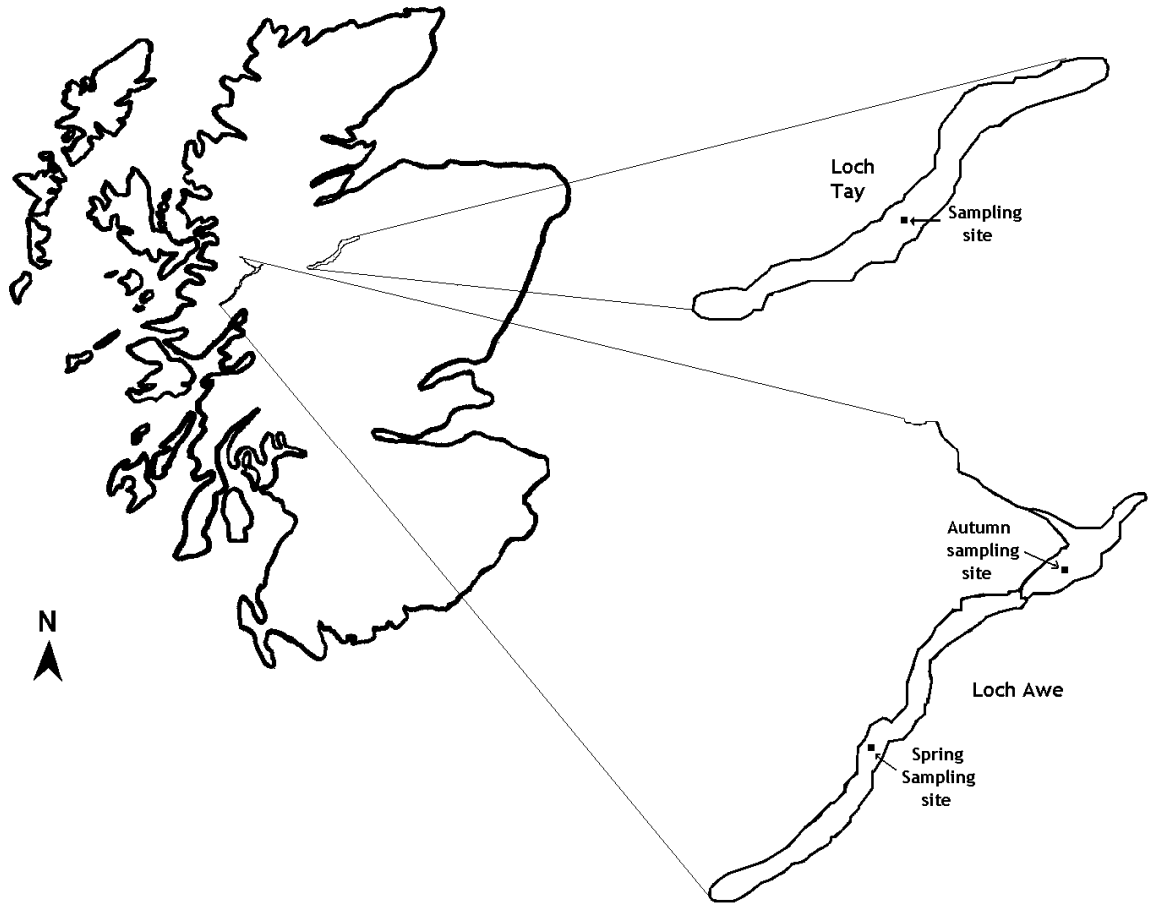

Fig. 5.1 Map of the geographic position of Loch Tay and Loch Awe in Scotland, and the location of sampling sites in each lake.

5.3.3. *Age and Growth parameters*

The surface of sagittal otoliths was ground, polished and examined according the technique of Fraser *et al*. (1998). Age was estimated by counting annuli. Three counts were performed and the final age determination was made by agreement of two independent readers. Growth of Arctic charr was expressed using the simplified Von Bertalanffy equation (von Bertalanffy, 1938) fitted to observed lengths at age using Marquardt least squares nonlinear regression:

$$L_t = L_\infty (1 - exp^{(-k(t-to))})$$

Where L_t = length at age t (annuli number), L_∞=maximum theoretical length, k=growth coefficient and t_0= the theoretical age at zero length. A two-parameter version of the Von Bertalanffy model (using k and L_∞) with the assumption that t_0=0, was applied. The non-linear estimation of growth parameters was calculated using the length-at-age data subroutine in FISAT II software (version 1.2.2, 2005), the length measure used was standard length (SL) throughout and the age was based on the number of annular rings observed in the otoliths. Subsequently, a multivariate maximum likelihood (ML) (Hesslein *et al.*, 1993) method was used to compare growth model estimates among phenotypes. This method tests the hypothesis of linear constraints on parameters that can be derived using the Likelihood ratios criterion which can be used when it is desired to test whether a sample came from a population with some "known" values for any or all of the parameters (L_∞, k, t_0). Linear constraints take the form of fixing any or all the parameters to their hypothesised values. When a single parameter is being tested it makes good sense to simply use a Z-statistic (since ML estimates are asymptotically normal). In this case, the degrees of freedom of X^2_r are equal to the number of parameters fixed (Kimura, 1980).

Likelihood Ratios were calculated in SPSS version 13 following the procedure described by Kimura (Kimura, 1980). A Von Bertalanffy growth curve was fitted and likelihood values were computed for each phenotype separately and the likelihood ratio statistic (χ^2) was then used to determine growth difference between phenotypes.

5.3.4. *Stable Isotope Analysis (SIA)*

From each fish collected in Loch Awe, two samples of white muscle (approximately 2cm by 2cm) were removed by dissection from the left flank below the dorsal fin and above the lateral line. The muscle samples were then placed into small plastic trays with labels and dried in an oven at 40^{o}C for 7 days. Each sample was ground into powder. Subsequently, samples of approximately 0.5 mg were placed in 5mm x 3mm tin capsules. For comparison, Loch Tay SIA results derived from Adams *et al* (2003) were used. Carbon and nitrogen stable isotope ratios were determined by continuous flow isotope ratio mass spectrometry at the Max Planck Institute for Limnology, Plön, Germany.

Stable isotope ratios are given using the δ notation expressed in units per mil (‰). Typical precision for a single analysis was ±0.1‰ for δ13C and ± 0.3‰ for δ15N. As lipids are depleted in ^{13}C, any variation in lipid concentrations between fish species could influence comparisons of δ^{13}C. This variation in δ^{13}C caused by lipid composition potentially complicates interpretation of dietary sources of carbon. A difference in lipid composition can give rise to variation in δ^{13}C values between individuals higher than the commonly assumed 1‰ difference between trophic levels, and hence may lead to biased interpretation of isotope results. Because of this problem, lipid-normalizing methods based on C:N ratios applicable to fish muscle sample are recommended to remove the effects of lipids. Therefore, here, fish data were arithmetically lipid-normalised (Kiljunen *et al*. 2006; Harrod & Grey, 2006). Also, non-parametric MANOVA was used to compare centroids location.

5.3.5. *Morphological analysis*

Landmark-based geometric morphometrics analyses were used to detect variation in the shape of individual charr. Photographs of the profile of the fish were taken by placing the animal on its right side in a fixed position with the tip of the mouth and the central part of the caudal fin along a straight line, using a Cannon digital camera (EOS 350D) fixed to a camera stand. The digital images were improved by adjusting brightness and contrast, using photo-editing software. Photographs of all fish analysed were first compiled using the computer program tpsUtil (Rohlf, 2006c). The scale factor on each image was set using the program tpsDig2 (Rohlf, 2006a). Then 28 landmarks (see Fig. 5.2) were defined on the body on each fish.

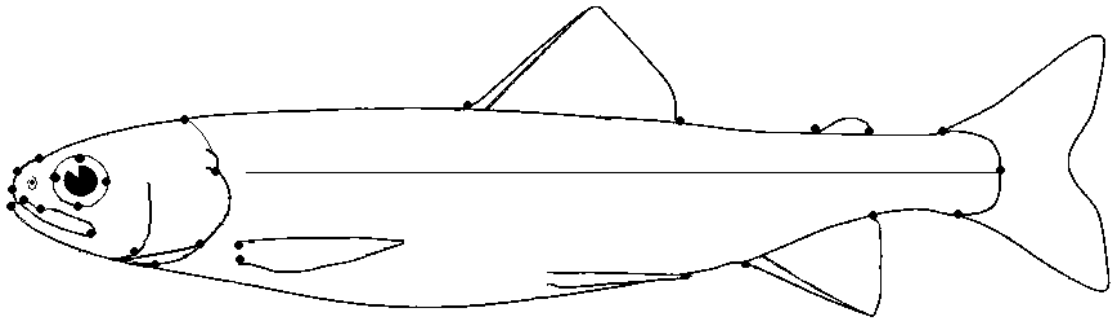

Fig. 5.2 Location of 28 anatomical landmarks used to define fish shape of spawning Arctic charr.

Generalized least squares (GLS) Procrustes superimposition was applied to the coordinates of raw landmarks to convert them into new shape variables called partial warps (PW). This method requires three steps: translation to a common origin, scaling to a common size and rotation to minimize summed squared inter-landmark distances among the forms (Rohlf & Slice, 1990). After superimposition the effect of size on shape is removed and both variables can be analyzed separately. The computer software tpsRelw (Rohlf, 2007) was used for this purpose.

Centroid size (CS) was used as a measure of overall body-size. It is defined as the square root of the summed, square distance of all landmarks about their centroid. CS exhibits all the desirable properties of a size variable, in particular that of being independent of shape under a null hypothesis of no allometry (Zelditch *et al.*, 2004).

The tpsRelw software was utilised to conduct a relative warp (RW) analysis (equivalent to principal component analysis) on the partial warps scores of each individual. RW scores were computed including the uniform component (which describes stretching or compression shape changes) using the algorithm given by Rohlf, (1996). The scaling option $\alpha=0$, to equally weight variation at scales of local deformation to find morphometric differences at all scales, was applied (Rohlf *et al.*, 1996). The program TwoGroup6 (Sheets, 2003) was used to obtain the mean shape differences among phenotypes within lochs, which were quantified from Procrustes coordinates using Goodall's F resampling test. Goodall's F test compares the Full and Partial Procrustes Distance between the means of two distinct groups and the amount of variance found within groups (Goodall, 1991; Adams *et al.*, 2004).

5.3.6. *Restriction Fragment Length Polymorphism and DNA isolation*

Mitochondrial DNA was extracted from adipose fin tissue. DNA isolation, amplification, and restriction enzyme analysis were carried out as described by (Knox *et al.*, 2002). Restriction fragment length polymorphism (Verspoor *et al.*, 1999) of the ND1, CYT B and D-Loop mitochondrial genes was applied to the amplified regions from the Polymerase Chain Reaction (PCR). Five restriction enzymes were used: Bcc1(Cyt-B and D-Loop), Hinf l and Mse l (Cyt-B), Dde l and Hae lll (Verspoor *et al.*, 1999). The resulting DNA fragments were separated on 2% agarose gels, stained with ethidium bromide, and visualised under ultraviolet light.

Variant fragment patterns were characterized with each restriction pattern given a single letter designation to generate a six letter composite haplotypes for each individual (Verspoor *et al.*, 1999). The genetic divergence among phenotype-defined populations was calculated using AMOVA analysis of the RFLP haplotypes using the software, Arlequin v.3.11.

AMOVA estimates the amount of genetic variation attributable to genetic differentiation among self-defined groups (F_{ct}), among populations within groups (F_{sc}), and among populations relative to the total sample (Ostbye *et al.*, 2005). The fixation index, F_{st}, is a measure of variance analogous to conventional F statistics and ranges from 0 to 1. High F_{st} implies a high degree of differentiation among populations. Euclidean distance matrix between pairs of haplotypes was used for the calculation of F_{st} values as an approximation of F-statistic (Weir & Cockerham, 1984). From a phylogenetic perspective, the entire mtDNA molecule is considered a supergene with numerous alleles, therefore in the input file for Arlequin 3.11, each restriction site was considered a distinct locus (see Table 1) although it is known that from a functional perspective mtDNA consists of 37 genes (Avise, 2004). Haplotype diversity (π) within and among *S. alpinus* samples was estimated by the average number of pairwise differences within and between populations (Nei & Li, 1979; Nei, 1987). Indirect estimates of gene flow or average migration rates ($Nm \approx (1-F_{st})/(4F_{st})$) were obtained from allele frequency differences based on F_{st} differences among phenotypes. The migration rate estimate is an average over the past tens to hundreds of generations. *Nm* was estimated by using the island model of migration (Allendorf & Luikart, 2007).

5.4. Results

In Loch Awe a total of 77 sexually mature fish were caught over the two sampling periods. In autumn, 43 mature individuals (33 males and 10 females) were collected and in the spring, 34 individuals (21 males and 13 females) were collected. All fish were collected from between 8.4 and 14.7 m depth. In addition 34 immature fish were collected (8 in autumn and 26 in spring). A total of 159 individual charr were captured in Loch Tay, from which 120 were sexually mature. Forty four mature fish of the small body-size phenotype were caught (24 males, 20 females) and for the large body-size phenotype 76 (39 males, 37 females) were collected. Sexually immature fish were not analysed further here.

5.4.2. *Age and Growth parameters*

Loch Awe, growth curves

The age of 44 sexually mature Arctic charr were determined, including 22 autumn and 22 spring spawners. Overall ages ranged from 2 to 5 years in spring spawners and from 2 to 6 in autumn spawners. Von Bertalanffy growth curves were fitted to length-at-age data (Fig. 5.3). The overall model did not show differences among spawning groups nor in the maximum theoretical length (L_∞) (p=0.07), however, the growth coefficient, *k*, was significantly higher in spring spawning charr (1.6) than in autumn spawners (0.2), (Table 5.1). The mean age at sexual maturity also differed among autumn spawners (4.7±0.24 years) and spring spawners (3.2±0.26 years), ($F_{1, 41} = 18.9$; P = 0.0009).

Table 5.1 Likelihood ratio tests comparing Von Bertalanffy parameter estimates for spring and autumn spawning Arctic charr (the total number of mean length at age values [N]=9), from Loch Awe.

Constraints	Spring $L_{\infty 1}$	Autumn $L_{\infty 2}$	Spring K_1	Autumn K_2	χ^2_r	d.f.	P
None	19.4	29.5	1.6	0.2			
$L_{\infty 1}$= $L_{\infty 2}$	20.2	20.2	1.1	0.6	3.3	1	0.07
K_1= K_2	20.8	21.2	0.6	0.6	3.7	1	0.05
All	20.2	20.2	0.6	0.6	4.4	3	0.2

Loch Tay, growth curves

The age range for large body-size charr was 2-5 and for the small body-size 2-7 years, but only a single age-7 individual was recorded. The mean age was not significantly different ($F_{1,38}$=0.001; p=0.97) between small body-size (3.63±0.89 years) and large body-size (3.62±1.39 years) phenotypes.

A Von Bertalanffy growth model was constructed using length-at-age of 44 charr (22 small body-size, 22 large body-size). Overall Von Bertalanffy models were significantly different between forms (Table 5.2). Not surprisingly the value of L∞ was significantly higher in the large body-size phenotype (L∞= 30.22) compared with the small body-size phenotype (L∞=17.04). In addition, k was higher for the large body-size phenotype (1.09) than for the small body-size phenotype (0.53), (Fig. 5.4).

Table 5.2 Likelihood ratio tests comparing Von Bertalanffy parameter estimates for small body-size and large body-size Arctic charr phenotypes (the total number of mean length at age values [N]=10) from Loch Tay.

Constraints	Small body-size $L_{\infty 1}$	Large body-size $L_{\infty 2}$	Small body-size K_1	Large body-size K_2	χ^2_r	d.f.	P
None	17.04	30.22	0.53	1.09	-	-	-
$L_{\infty 1}$= $L_{\infty 2}$	35.9	35.9	0.12	0.55	29.2	1	<0.0001
K_1= K_2	21.9	48.25	0.26	0.26	33.2	1	<0.0001
All	35.9	35.9	0.26	0.26	53.9	3	<0.0001

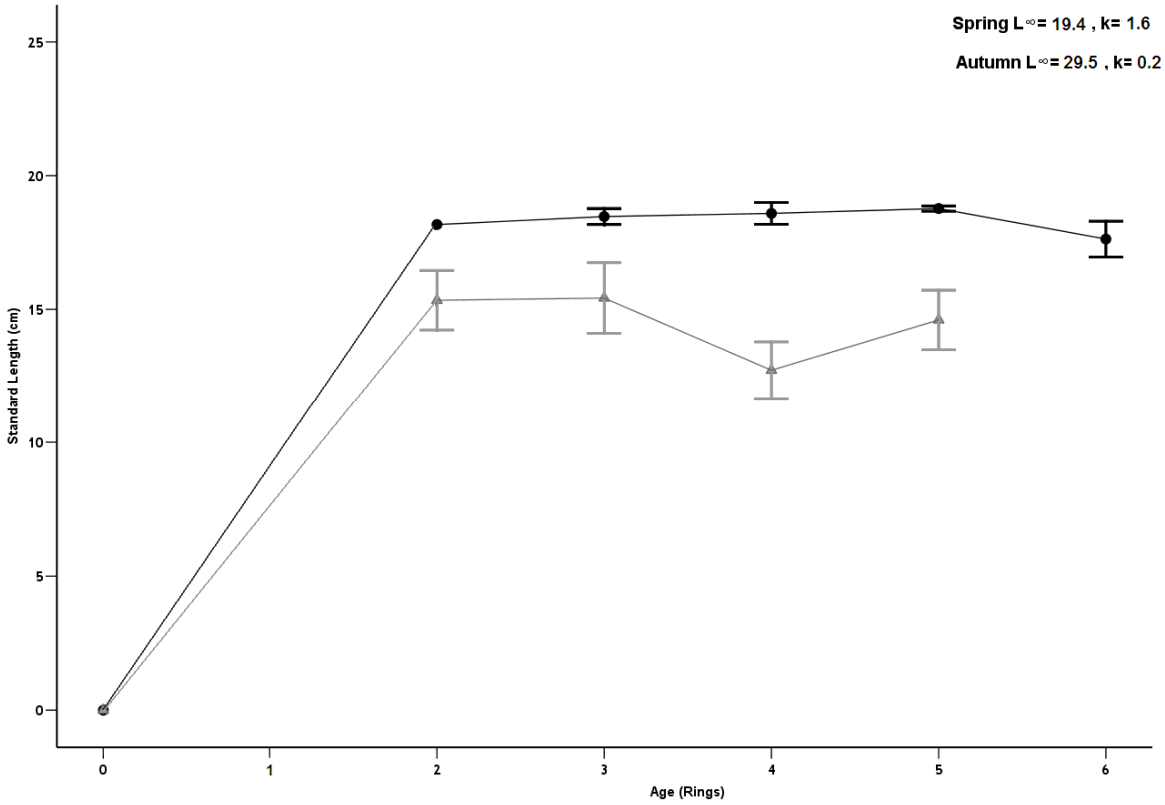

Fig. 5.3 Growth curve of mean ±S.E. length by age obtained using a Von Bertalanffy model fitted to spring and autumn Arctic charr spawning phenotypes from Loch Awe.

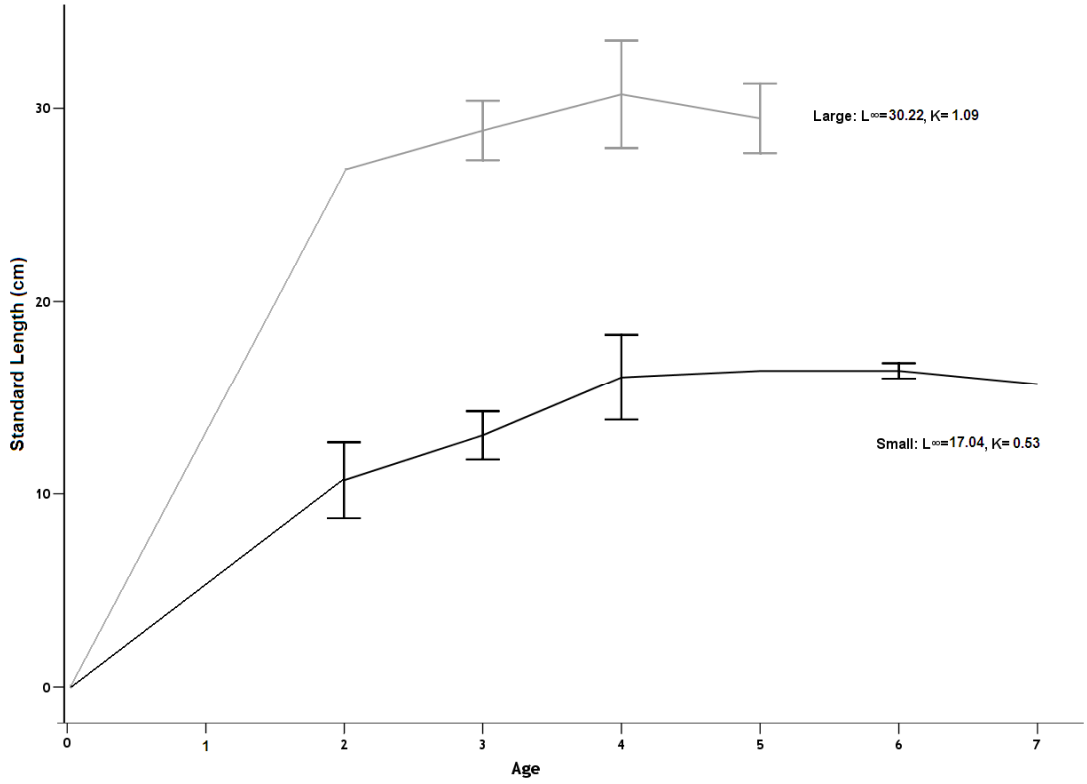

Fig. 5.4 Growth curve of mean ±S.E. length by age obtained using a Von Bertalanffy model fitted to Arctic charr in Loch Tay small body-size spawning phenotype, compared to the large body-size spawning phenotype.

5.4.3. *Stable Isotope Analysis (SIA)*

a) *Loch Awe*

Loch Awe sexually mature fish collected from autumn and spring spawning periods differed in mean nitrogen stable isotope ratios ($\delta^{15}N$ for autumn 10.8± 0.26 ‰ and 13.7± 0.4 ‰ for spring; t_{33}=-6.46; p<0.0001; Fig. 5) and mean carbon stable isotope ratios ($\delta^{13}C$ for autumn -29.9± 0.31 ‰ and -28.4± 0.3 ‰ for spring; t_{33}=-3.16; p<0.003; Fig. 5). Also, mean C:N ratio (a correlate of lipid concentration) was significantly different between phenotypes ($F_{1,34}$=12.5, p=0.001). The autumn spawners had considerably depleted $\delta^{15}N$ and $\delta^{13}C$ values compared with spring spawners.

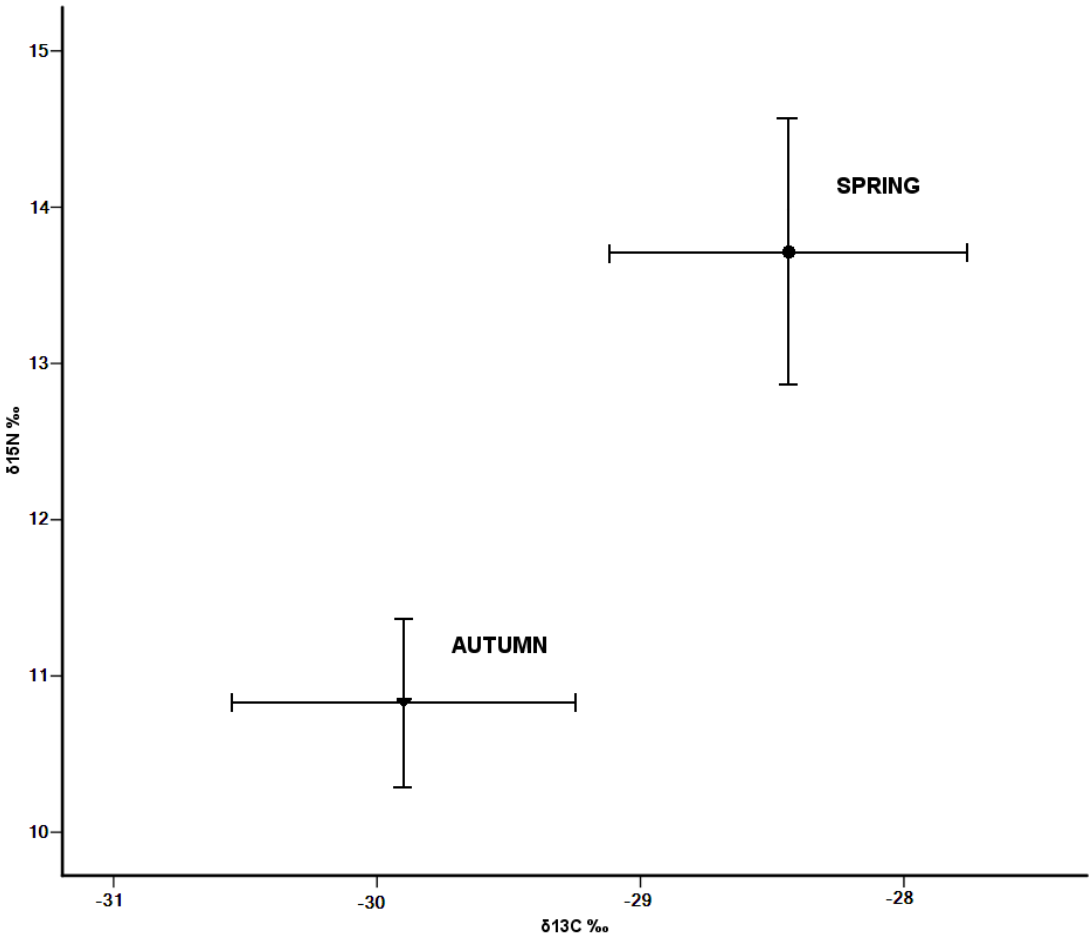

Fig. 5.5 Variation in mean (±S.E). $\delta^{13}C$ and $\delta^{15}N$ values of white muscle of autumn spawning and spring spawning Arctic charr collected from Loch Awe.

Loch Tay

Data for charr phenotypes from Loch Tay were extracted from Adams (2003a), these data showed discrete segregation among modal size groups, the lower mode Arctic charr (small body-size phenotype) had very significantly higher mean $\delta^{15}N$ and $\delta^{13}C$ signatures than the upper mode (large body-size phenotype), ($\delta^{15}N$, lower mode 11.9±0.2‰; (mean±S.E.) *cf.* upper mode, 10.7±0.1‰; t=5.48, d.f.=71, P=0.00001) ($\delta^{13}C$; lower mode -26.6±0.2‰ *cf.* upper mode, -27.2±0.1‰; t=2.86, d.f.=71, P=0.006) .

5.4.4. *Morphological analysis*

Loch Awe

Generalized Goodall's F resampling test showed significant differences in mean overall shape between the two charr spawning groups from Loch Awe ($F_{52, 3432}$ = 2.65; p=0.00001) and a Partial Procrustes distance (indicative of overall shape difference) between means of 0.0137. Centroid size (CS), was not significantly different between forms (autumn spawning phenotype=35.25±6.5 and spring spawning phenotype =32.15±6.4; p=0.072). Together, the first three main relative warps together explained 49% of the overall shape variation. An analysis of variance showed that there was a highly significant difference between spawning phenotypes in RW1 and RW3 score means. In addition RW1 scores were significantly different between sexes (Table 5.3). However, RW2 score means were not significantly different for either sex or phenotype.

Table 5.3 Multivariate tests of Relative Warps among Phenotype, Sex and the interaction of both factors for fish from Loch Awe. p is the F-test significance of each Relative Warp.

Relative Warp	Variance Explained %	*p*		
		Phenotype	Sex	Phenotype* Sex
1	26	0.010	0.0001	0.0001
2	14.5	0.2	0.4	0.4
3	8.5	0.004	0.2	0.004

Figure 5.6 shows shape variation for RW1 and RW3 scores in both spawning phenotypes. Graphic representations of the most extreme negative and positive values of each axis show shape variation present on the head and the body. RW1 positive scores indicate more robust body, rounded and elongated snout, the head and the eye are enlarged, whereas, negative values depict fusiform and thin fish with reduced head and shorter snout. RW1 scores also describe significant differences among sexes. Charr with high positive RW3 scores typically exhibited a very sharp and elevated snout, as well as deeper body; in contrast, individuals with negative scores have protuberant snout curvature and reduced caudal peduncle (Fig. 5.6).

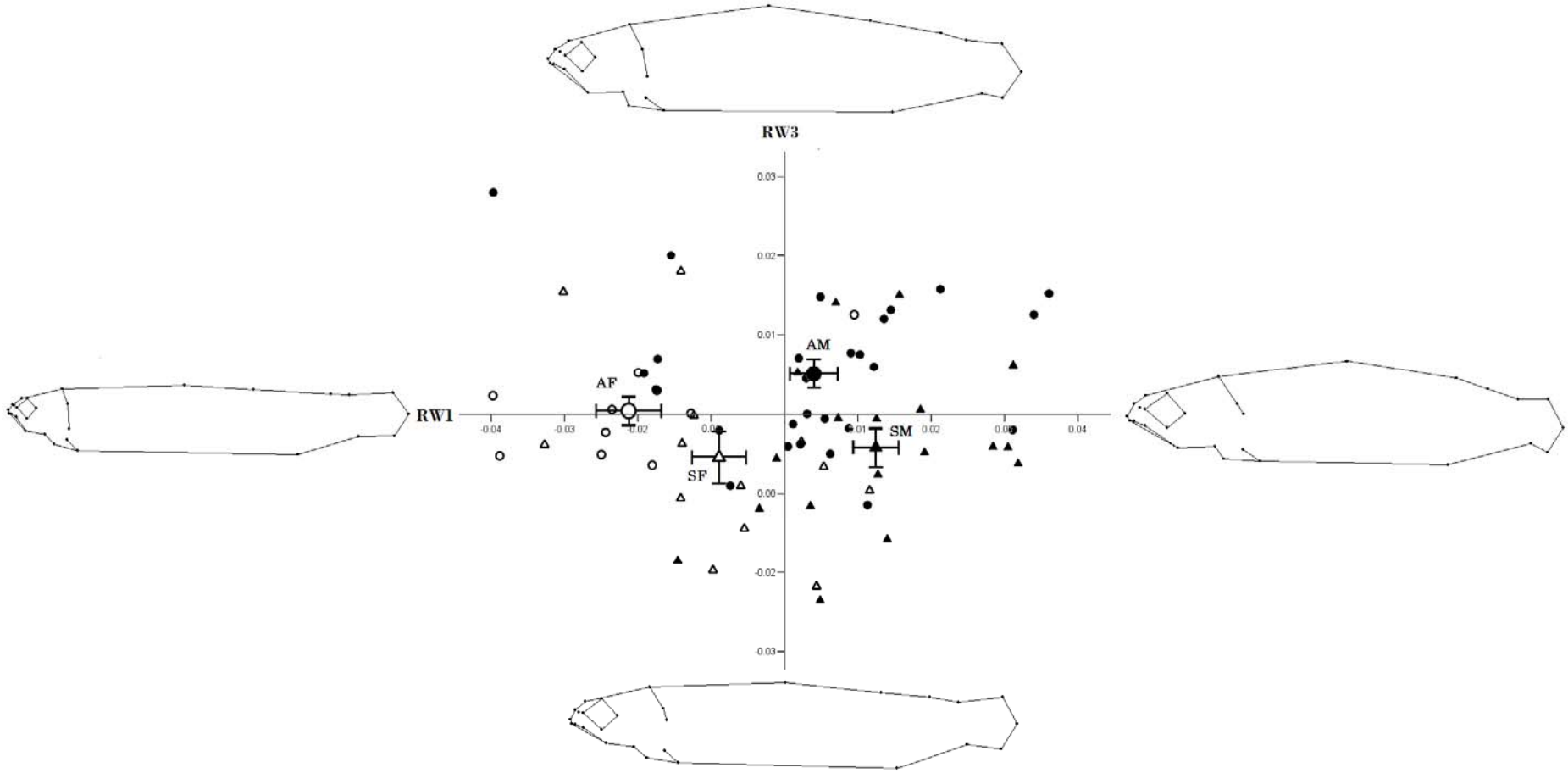

Fig. 5.6 RW1 and RW3 scores of autumn spawning and spring spawning charr from Loch Awe, plus mean ±S.E. Graphic representations are illustrated showing the most extreme negative and positive values of each axis defined as deviates from the pool mean shape represented by the origin of the scatterplot. Mean ±S.E. scores of autumn spawning phenotype males (AM) and females (AF) and spring spawning phenotype males (SM) and females (SF) are shown. Landmarks are connected by lines to facilitate the visualization of the shapes

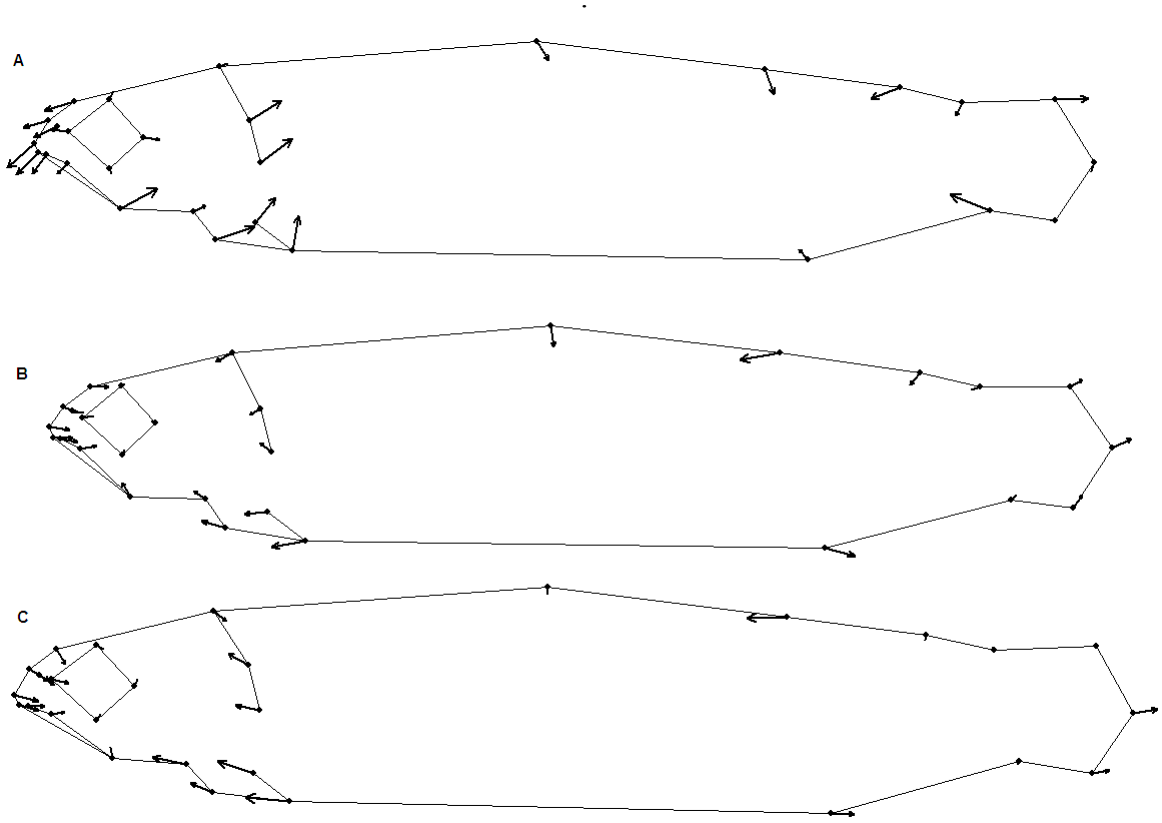

Fig. 5.7 Shapes of the autumn spawning and the spring spawning phenotypes of Loch Awe. In **A** landmarks indicate the autumn spawning phenotype shape and vectors indicate the spring spawning phenotype shape as a deformation from autumn spawning phenotype shape. In **B** vectors represent the shape of the autumn spawning females as deformation from autumn spawning males shape (landmarks) and in **C** vectors represent spring spawning females shape as deformation from spring spawning males (landmarks). Landmarks are connected by links to facilitate the visualization shape.

Comparisons of the actual mean shapes between spawning groups are depicted in Fig. 5.7. The autumn spawning phenotype (Fig. 5.7A) is depicted by the landmarks and the vectors displayed, from them represent the shape of the spring spawning phenotype. The spring spawning charr phenotype is characterised by a more robust and longer head, the snout landmarks move anteriorly and the opercular bone landmarks move posteriorly, also they show a bigger eye and longer lower jaw, the jaw articulation point moves posteriorly, the maxillary bone is notably longer and elevated, making the anterior head shape looking robust, the top of the mouth is positioned more ventrally and the caudal peduncle is narrower than in the autumn spawning phenotype.

Figure 5.7 also depicts mean shape of males and females of each phenotype. The autumn spawning males (landmarks, 5.7B) and spring spawning males (landmarks, 5.7C) have more robust head, bigger eye and deeper body than autumn spawning females (vectors, 5.7B) and spring spawning females (vectors, 5.7C) respectively.

Loch Tay

A discrete body-size variation was found in charr from Loch Tay. The overall body means shape (including all partial warps scores) was highly significantly different among phenotypes (Generalized Goodall's $F_{42, 4956} = 71.9$; $p < 0.00001$).

A conventional F-test was used to examine the effect of large and small body-size phenotype and sex on the first three relative warps. Relative warps 1, 2 and 3 account for 43, 15 and 10% of variation respectively. RW1 and RW3 scores showed significant differences for both phenotype and sex. However, RW2 was not significantly different for either sex or phenotype (Hesslein *et al.*, 1993).

Fig. 5.4 Multivariate tests of Relative Warps among Phenotype, Sex and the interaction of both factors for fish from Loch Tay. p is the F-test significance of each Relative Warp.

Relative Warp	Variance Explained %	*p*		
		Phenotype	Sex	Phenotype* Sex
1	41.4	**0.0001**	**0.0001**	**0.001**
2	15.3	0.3	0.4	0.78
3	9.3	**0.04**	**0.0001**	**0.01**

The individual RW1 and RW3 scores for each fish are presented in Figure 5.8, which illustrates the shape deformation from the pooled mean to the negative or positive extremes for the first and the third relative warps. In RW1, negative scores represent a more slender body shape than the opposite scores; however, the converse is true for the head landmarks. For extreme negative RW1 scores, the tip of the snout and the nostril move anteriorly; at the same time the area of the eye is increased with respect to the head area, meanwhile, the operculum landmarks displace their position in an opposite direction to the snout. Thus

highly negative RW1 scores represent a thin body shape with a robust head and prominent eye. On the other hand, highly positive RW1 scores, describe a shape of fish with a robust body, a sharp snout curvature, protruding lower jaw, the tip of the snout pointed upwards, a smaller eye and the position of the dorsal fin extended vertically and a head reduced in proportion to the body. RW1 captures most of the variation between phenotypes; therefore both phenotypes are clearly segregated along this axis.

Figure 5.9A shows comparisons between the actual mean shapes of small body-size and large body-size phenotypes from Loch Tay. Small body-size phenotype fish, depicted by the vectors shown, have a bigger eye, bigger head, thinner and more fusiform body with the top of the mouth positioned more ventrally relative to fish with the large body-size phenotype (represented by landmarks).

Figure 5.8 also depicts the variation in RW3 scores, which also shows sexual dimorphism within phenotypes. Figure 5.9B shows comparisons between the actual mean shapes of sexes within phenotypes. Small body-size females (landmarks) have a smaller head, smaller lower jaw and shorter maxillary bone in comparison with the small body-size males (vectors display), no significant changes are present neither in the eye nor in the body shape. Meanwhile, the large body-size males (vectors shown), show a larger head, larger lower jaw, larger maxillary bone and deeper body by comparison with large body-size females (landmarks), no significant changes are present in the eye shape.

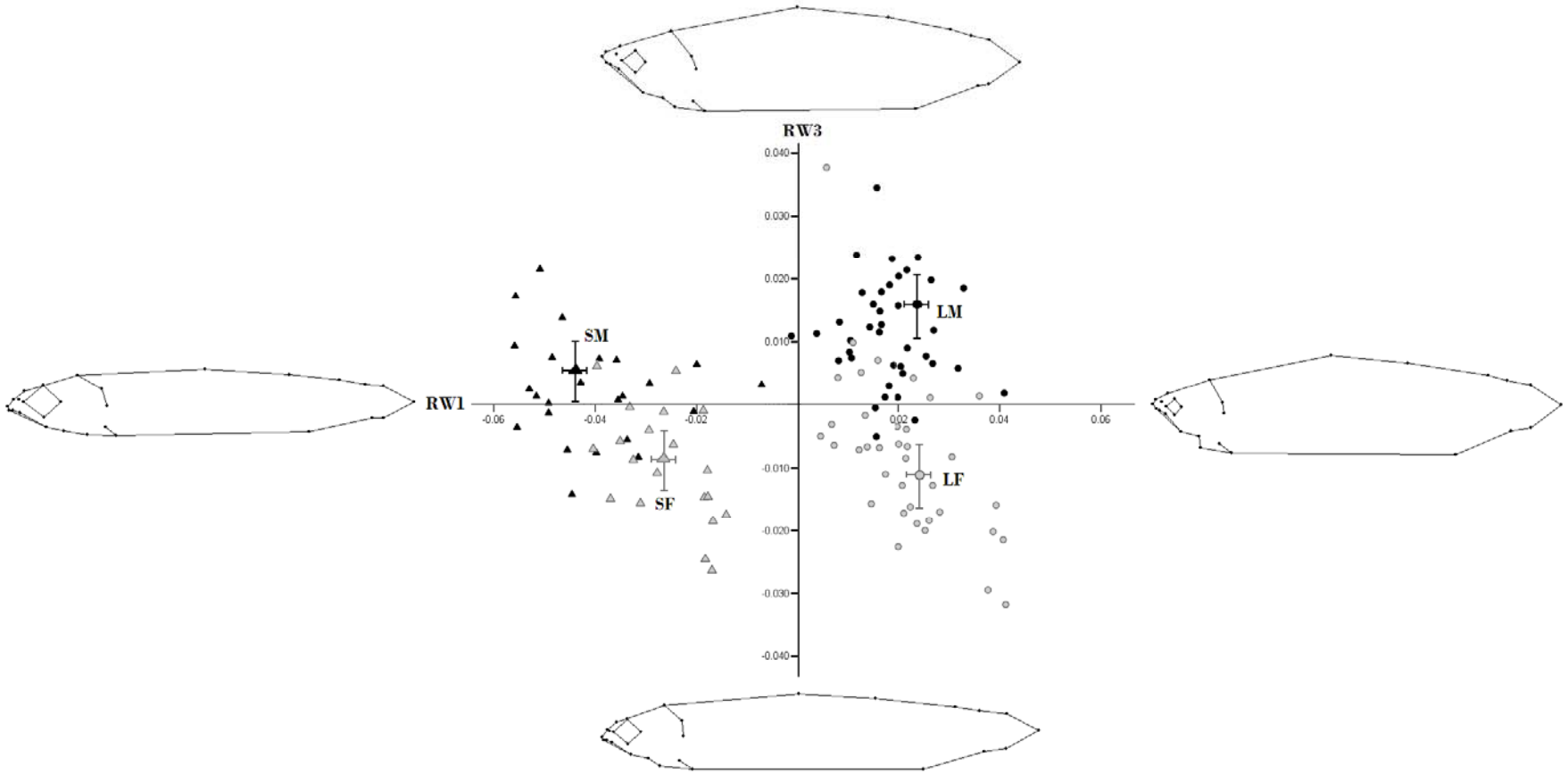

Fig. 5.8 RW1 and RW3 scores of sexually mature Arctic charr from the large body-size and small body-size groups collected in Loch Tay during spawning period. Mean ±S.E. scores of large body-size males (LM) and females (LF) and small body-size males (SM) and females (SF) are shown. Landmarks are connected by lines to facilitate the visualization of the grand mean shape.

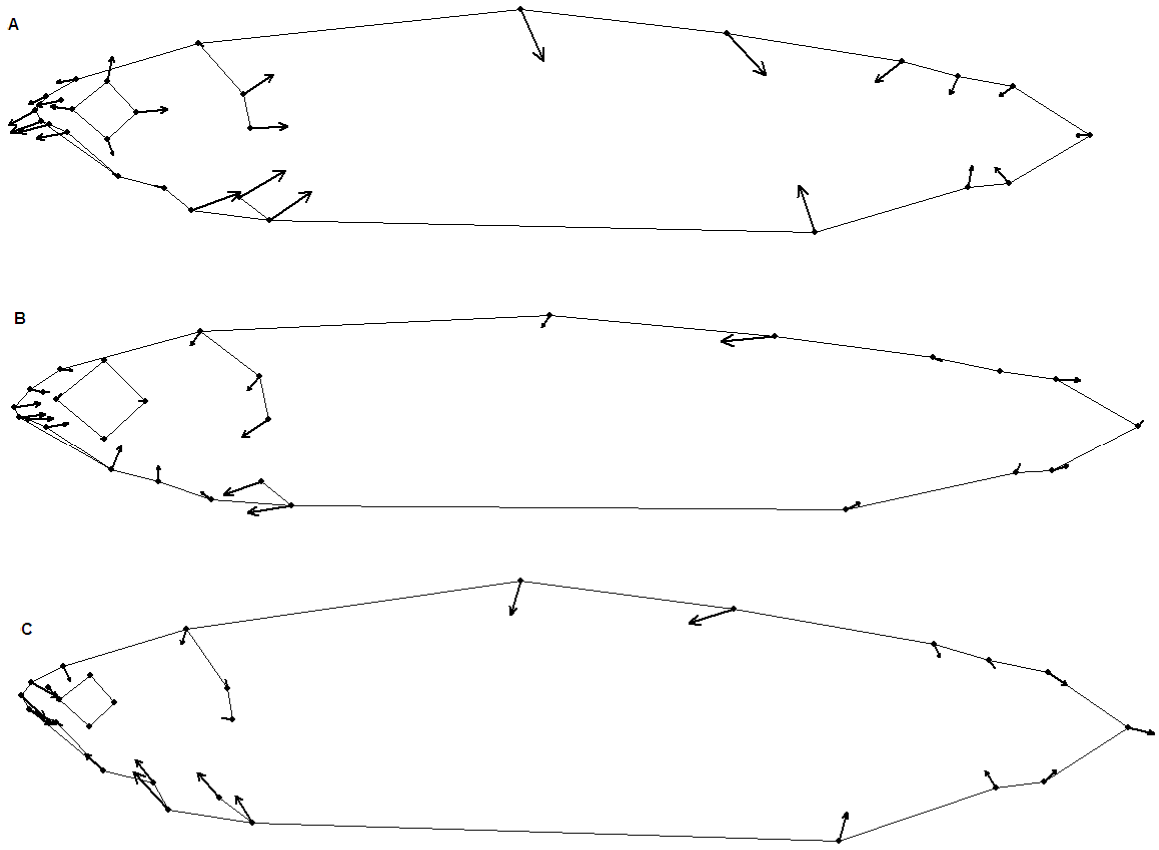

Fig. 5.9 Shapes of the small body-size and the large body size spawning phenotypes from Loch Tay. In **A** landmarks indicate the large body size-phenotype shape and vectors indicate the small body-size phenotype shape as a deformation from large body-size phenotype shape. In **B** vectors represent the shape of the small body-size females as deformation from small body-size males shape (landmarks) and in **C** vectors represent large body size females as deformation from large body-size males (landmarks). Landmarks are connected by links to facilitate the visualization shape.

5.4.5. *Mitochondrial DNA Analysis*

a) *Loch Awe, genetic variation*

Four enzymes: *BccI, HinfI, MseI* and *DdeI*, showed differences in cleavage patterns which were considered genetic polymorphism. A total of three composite haplotypes was revealed with the RFLP analysis performed on the ND-1, Cyt B and D-Loop genes (Table 5.5). The three haplotypes were present in both phenotypes (Table 5.6).

For mature individuals the AMOVA analysis revealed no significant difference in haplotypes frequencies between spawning groups ($Fst_{1,109}$= 0.03; p=0.1, see Table 7). This analysis showed that almost all the variation in mtDNA occurred within phenotypes (97 %), meanwhile variation among subpopulations was very low (3%). Haplotype diversity within pooled samples was higher in spring spawners (0.44 ±s.d. 0.09) than in autumn spawning spawners (0.17 ±s.d. 0.07). Moreover, estimated average migration rates (*Nm*≈8.1) suggests that 8 individual per generation could potentially interbreed between phenotypes.

Loch Tay, genetic variation

A total of four composite haplotypes were present in both phenotypes of charr from Loch Tay (Table 5.5). In the present analysis both phenotypes revealed two haplotypes in common (I and II) while the remaining two (III and IV) were present in low frequency and were only recorded from the small body-size phenotype (Table 5.6).

Table 5.5 Variant restriction patterns showing the four Arctic charr mtDNA haplotypes generated by restriction enzymes. Haplotypes are numbered by ranking in alphabetical order the digestion types of each restriction endonuclease.

		D-Loop	CYT B			ND1	
Haplotypes	**Loch**	**Bcc1**	**Hinf I**	**Mse l**	**Bcc l**	**Dde l**	**Hae lll**
I	Tay/Awe	A	A	A	B	B	B
II	Tay/Awe	A	A	A	B	A	B
III	Tay	B	B	B	B	A	B
IV	Tay	B	B	B	B	B	B
V	Awe	A	C	A	B	B	B

AMOVA detected a significant overall Fst (0.39; p<0.001) when comparing mtDNA genetic variation among phenotypes (see Table 5.7). AMOVA also revealed that the majority (60.2%) of mtDNA variation in charr tested here occured within phenotypes, but a significant portion (39.8%) was attributable to differences among phenotypes. Average haplotype diversity of the pooled samples was higher (2.9 ±s.d. 1.5) in the small body-size phenotype and lower (0.17 ±s.d. 0.1) in the large body-size phenotype, indicating greater haplotype diversity of the small body-size charr. Indirect measures of gene flow (Nm)

showed very low value, indicating that less than one fish (0.4 immigrants per generation) is interbreeding between phenotypes.

Table 5.6 Distribution of Arctic charr spawning subpopulations mtDNA D-Loops, Cyt b and ND-1 haplotypes and their relative frequencies in the populations studied. Five haplotypes were observed.

Phenotypes	N	Haplotypes				
		I	II	III	IV	V
		AAABAB	AAABBB	BBBBAB	BBBBBB	ACABBB
Tay Small body-size	42	0.36	0.45	0.17	0.024	-
Tay Large body-size	76	0.03	0.97	0	0	-
Awe Autumn	43	0.07	0.91	-	-	0.02
Awe Spring	34	0.12	0.73	-	-	0.15

Fig. 5.7 AMOVA of the mitochondrial DNA data by phenotype in each lake

Source of Variation	d.f.		Sum of squares		Variance components		Percentage of total variation	
	Loch Tay	Loch Awe	Loch Tay	Loch Awe	Loch Tay	Loch Awe	Loch Tay	Loch Awe
Among phenotypes	1	1	25.7	0.67	0.4	0.01	39.8	2.99
Within phenotypes	127	75	77.7	23.1	0.6	0.31	60.2	97.1
Total	128	76	103.5	23.8	1.02	0.32		
Fixation index (F_{st})							**0.39**	**0.03**

5.5. Discussion

5.5.2. *Loch Awe*

The analysis of stable isotopes in muscle tissue provides an estimation of long-term (> 6 months) assimilated food intake (Hesslein *et al.*, 1993). Analysis of $\delta^{13}C$ has been shown to differentiate carbon emanating from littoral (near shore) production (benthic algae and allochthonous sources) and pelagic production (from phytoplankton). The $\delta^{13}C$ of the base of the littoral food web tends to be enriched in ^{13}C (less negative $\delta^{13}C$) relative to the base of the pelagic food web (France, 1995b). $\delta^{13}C$ can be used to indicate ultimate carbon source in consumers because $\delta^{13}C$ of consumers is related to that of their food (France, 1995a; 1995b). The ratio of stable isotopes of nitrogen ($\delta^{15}N$) provides information on trophic position as the $\delta^{15}N$ of a consumer is typically enriched by 3-4‰ relative to its diet (Vander Zanden & Rasmussen, 1999; Post *et al.*, 2000; Post, 2002). The results of this study show that the two spawning phenotypes (autumn and spring) of Loch Awe differ significantly in trophic ecology. Both $\delta^{13}C$ and $\delta^{15}N$ differed significantly between the two spawning phenotypes from Loch Awe indicating dietary segregation and foraging habitat use differences and that the two spawning phenotypes forage at different trophic levels. The spring spawning phenotype had a $\delta^{15}N$ of 2.9‰ higher than the autumn spawning phenotype, assuming that nitrogen baseline is similar in both foraging habitats; this suggests that the spring spawning phenotype is foraging on average approximately one trophic level higher. The most likely explanation for this is that autumn spawning fish feed on plankton in the pelagic zone, whilst spring spawning fish feed on macro-benthic prey (littoral zone) (Vander Zanden *et al.*, 2005).

Significant differences in the morphology of the Loch Awe phenotypes appear to be related to foraging. The spring spawning phenotype exhibit many characteristics typical of macro-benthic feeding fish; robust and longer head, longer lower jaw, longer maxillary bone and the top of the mouth is positioned more ventrally. However, spring spawning fish have a less robust body than the autumn spawning phenotype fish. On the other hand, the autumn spawning phenotype showed characteristics typical of planktivorous fish; a shortened and thin head, reduced eye, shorter jaw length, shorter maxillary bone and narrower caudal peduncle (McPhail, 1992; Walker, 1997; Bertrand *et al.*, 2008).

There was also clear evidence of differences in life history features between the two phenotypes from Loch Awe. Possibly as a consequence of different feeding ecology, the growth rates of the groups differed. The spring spawning phenotype individuals grew faster and matured younger than the autumn spawning phenotype, although they are not different in size.

Thus the two spawning phenotypes showed clear ecological, phenotypic and life history segregation, but no clear evidence of gene pool segregation on the basis of the mtDNA markers used in this study. Despite the lack of mtDNA evidence of differences, gene flow between phenotypes is very unlikely given the temporal segregation in their spawning periods. A parsimonious explanation is that the two spawning phenotypes are genetically very closely related and that spawning segregation is a very recent divergence.

5.5.3. *Loch Tay*

The two phenotypes of Arctic charr from Loch Tay clearly differed in feeding ecology. Differences in mean $\delta^{13}C$ and $\delta^{15}N$, indicate at least partial dietary segregation and utilisation of alternative foraging niches. Charr form Loch Tay also differed in morphological characteristics. Between form shape variations show an interesting pattern. On the one hand, the head shape of the small body-size phenotype appears to be typical for small benthic fed fish: longer and wider jaws, longer head and large eye size, blunt snout and sub-terminal mouth (Snorrason *et al.*, 1994; Adams *et al.*, 1998; Adams *et al.*, 2003b). However these charr do not showed a heavy robust or stocky body as described for other benthic charr in Scotland (Walker *et al.*, 1988; Adams *et al.*, 1998), but a streamlined one. In contrast, fish of the large body-size phenotype showed similar morphology to that described for planktivorous phenotypes with pointed snouts and protruding lower jaws (Snorrason *et al.*, 1994), moreover, the snout is upwards, the eye is small like pelagic individuals (Adams *et al.*, 1998), but, the body is more robust and heavy. The results of the SIA support the conclusion that the large body-size charr are planktivorous feeders (having lower $\delta^{13}C$ and $\delta^{15}N$ values) and the small body-size fish are macro-benthos feeders (higher $\delta^{13}C$ and $\delta^{15}N$ values).

In addition, strong evidence of life history variation is shown here. The two charr phenotypes grew at different rates; as expected, the growth coefficient (K) value was greater for the large body-size phenotype was greater. Variation in growth appears to be a

consistent feature of these populations because the bimodal body-size distribution has been maintained for at least the last 8 years (cf. Adams *et al.*, 2003a).

The greater genetic variation based on the relative high value (Wilson *et al.*, 2004) and very high significance ($p<0.0001$) of the genetic differentiation (Ostbye *et al.*, 2005) plus the elevated haplotype diversity and very low *Nm* values (0.4) and the presence of private alleles indicate that these two group are operating as two distinct gene pools with no effective gene flow between them (Adams *et al.*, 2006) .

The results of this study show similarities and significant differences between the contrasting sympatric systems, and the observed patterns are suggestive of the ecological and evolutionary mechanisms that gave rise to these systems. Both show clear evidence of trophic segregation between forms. In both systems a plankton feeder and a macro-benthos feeder are sustained, and although these may be some dietary and spatial foraging overlap, to a large extent these groups are ecologically segregated.

There were also similarities between sites in morphological characteristics. The groups with stable isotope values (Loch Tay: large body-size, Loch Awe: autumn spawning phenotype) indicative of planktonic foraging had a morphology also indicative of plankton feeding. This was also true for the groups with SIA signatures indicative of littoral foraging (Loch Tay: small body-size, Loch Awe: spring spawning phenotype).

In both systems there is evidence that the two forms are operating as separate gene pools. In Loch Tay this is shown by very significant differences in a suite of non selective mtDNA marker, showing haplotype frequency differences, private alleles and low between phenotypes *Nm* estimates. In Loch Awe there are no clear differences in the mtDNA markers, rather gene pool segregation is inferred from the temporal segregation between groups at spawning. This pattern strongly suggests that the loch Awe forms segregated very recently and most likely while in sympatry but that the Loch Tay forms represent a more ancient segregation (pre-glacial) potentially (but not certainly) post glacial invasion.

ARCTIC CHARR FROM LOCH TAY

Large body-size phenotype

Small body-size phenotype

ARCTIC CHARR FROM LOCH AWE

Spring spawning phenotype

Autumn spawning phenotype

CHAPTER 6. VARIATION IN SCALE SHAPE AMONGST ALTERNATIVE SYMPATRIC PHENOTYPES OF ARCTIC CHARR *SALVELINUS ALPINUS* FROM TWO LAKES IN SCOTLAND

*** Note: This chapter has been submitted as a manuscript to the "Journal of Fish Biology".**

6.1. Introduction

The coexistence of individuals of the same species expressing more than one discrete, alternative phenotype for a given characteristic represent an important step in the process of divergence which may lead to genetic segregation and ultimately speciation. This is because the expression of sympatric alternative phenotypes provides alternative phenotypic modes upon which selection can act independently, thus enabling diversifying selection (West-Eberhard 1989; 2003). Such alternative phenotypes appear to have evolved relatively frequently in sympatry in freshwater fishes inhabiting postglacial lakes (Schluter & McPhail 1992; Alexander & Adams, 2000; Robinson and Parsons, 2002).

In many species discrimination between one morph and another can be difficult, therefore is helpful to look for methods to detect phenotypic variation in specific traits. The morphological characteristics of fish scales have proved to be useful tool to discriminate species of the same genus, populations of the same drainage basin (Jawad & Al Jufaili, 2007; Poulet et al., 2005) and identify spawning stocks (Margraf & Riley, 1993; Watkinson & Gillis, 2005). There have been attempts to use the shape of scales to discriminate between closely related fish species and between stocks of the same species. These have mostly focused on the use of relatively complex Fourier analyses of shape with variable success (Pontual and Prouzet, 1987; Margraf & Riley, 1993; Poulet et al., 2005). Recently Ibañez et al. (2007) used the more accessible geometric morphometric analysis to show that scale shape was a good discriminator of genera and species within the Mugilidae, but, this study indicated that discrimination at the population level was least effective in discriminating populations from nearby areas. More recently, Ibanez et al. (2009) found that also scale shape varies between anatomical regions of the fish that maybe related with the swimming mode of the species. This study extends the use of scale variation to test for differences in scale shape between ecologically distinct alternative phenotypes of Arctic charr living in sympatry. Arctic charr is predominantly a freshwater species in which sympatric alternative phenotypes are common and frequently display

different morphological characteristics (see Jonsson and Jonsson, 2001 for a review). Both Loch Awe and Loch Tay support populations of sympatric alternative phenotypes (polymorphic sensu Smith & Skulason, 1996) of lacustrine Arctic charr. Loch Tay charr exhibit a bimodal size-frequency distribution amongst sexually mature fish at spawning time (in autumn); both males and females ranging from 80 to 160 mm (small body-size) and 190 to 290mm (large body-size) in fork length (FL) (Adams et al., 2003). In Loch Awe, sexually mature charr individuals are unimodal in body size, but segregate into two distinct spawning groups; those that spawn in spring and those that spawn in autumn (Alexander & Adams, 2000; Kettle-White, 2001). There is evidence of body shape differences between sympatric phenotypes within lochs at both sites (see chapter 5), however these differences are relatively subtle compared with those of many other sympatric Arctic charr phenotypes (Adams et al., 1998; Eiríksson et al., 1999; Fraser et al., 1998; Skúlason et al., 1996; Snorrason et al., 1994).

6.2. Materials and Methods

6.2.2. *Study areas and sampling*

Sexually mature Arctic charr were collected by standard Nordic mono-filament survey gill-nets, from spawning sites at spawning time in Loch Tay (56°30′ N; 004°10′ W) east central Scotland (October) (large body-size N=20, small body-size N=14) and Loch Awe (56° 20′ N, 005° 05′W) west central Scotland during November (autumn, N=18) and February (spring, N=10).

Scales were removed from the flank immediately anterior to the dorsal fin and photographed with a camera (JVC model TK-C1381) mounted on a dissecting microscope. Shape was analysed using landmark-based geometric morphometric methods (Rohlf, 1990). The digital images were first compiled using the computer program tpsUtil (Rohlf, 2006b). The scale factor on each image was set using the program tpsDig2 (Rohlf, 2006a). Nine landmarks were defined and located on one scale from each fish (Fig 6.1). Generalised least squares Procrustes superimposition (GLS) was applied to the coordinates of raw landmarks to convert them into new shape variables, (partial warps (PW)), independent of the scale size (Rohlf, 1990). These were then analysed for shape differences using a Goodall's F-test. The tpsRelw program (Rohlf, 2007) was used to run a Relative Warp Analysis, (similar to a Principal Component Analysis) on the covariance

matrix derived from the partial warp scores, this analysis is used to describe the main shape variation. The centroid size (CS), defined as the square root of the summed square distance of all landmarks about their centroid (Zelditch, 2004) and was calculated as a measure of overall scale size.

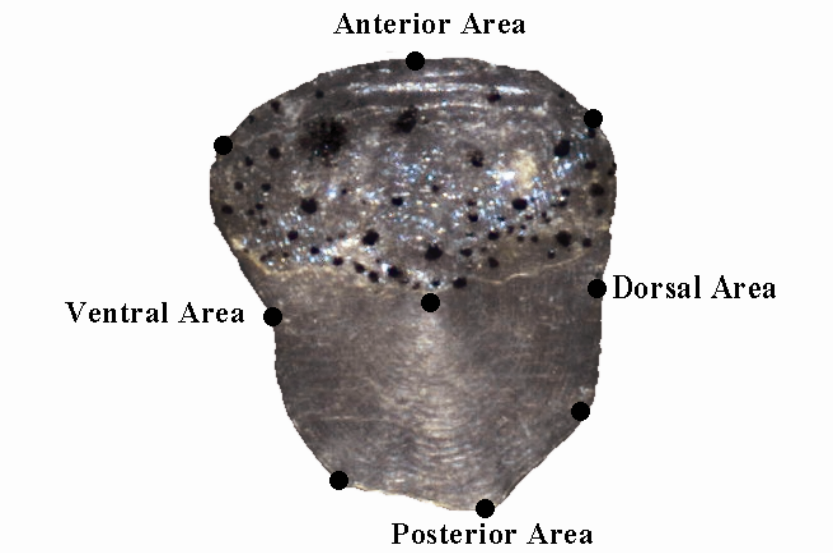

Fig. 6.1 Landmarks used to define the shape of the scales. The areas of the scales are described with respect to the fish position.

6.3. Results

Landmark-based geometric morphometrics successfully detected differences in scale shape between ecologically distinct populations of S. alpinus living in the same loch. Sympatric phenotypes from Loch Tay and Loch Awe showed clear variation in the morphology of their scales. Although the population sample size was relatively small, results were highly significant.

Overall, the shape of scales from the two phenotypes of S. alpinus from Loch Tay differed significantly (comparison of all partial warp scores $F_{14,448}= 5.7$; $p = 0.0001$). Relative warp 1 (RW1) explained 47 % of the total variation in shape of the scales of charr from Loch Tay. RW1 scores differed significantly between the two sympatric phenotypes from Loch Tay ($F_{1,33}=10.2$; $p=0.003$). The consensus shape of scales of the large and small body-size phenotypes is shown in Fig. 6.2. The large body-size phenotype scales were broad and round with the anterior edge highly reduced in comparison to the scales of the small body-size phenotype which are long and thin. The centroid size was also,

significantly different (F1,33=292.5; p=0.0001), the large body-size phenotype had larger scales (2.8+0.34 mm) than the small body-size phenotype (1.2+0.18mm).

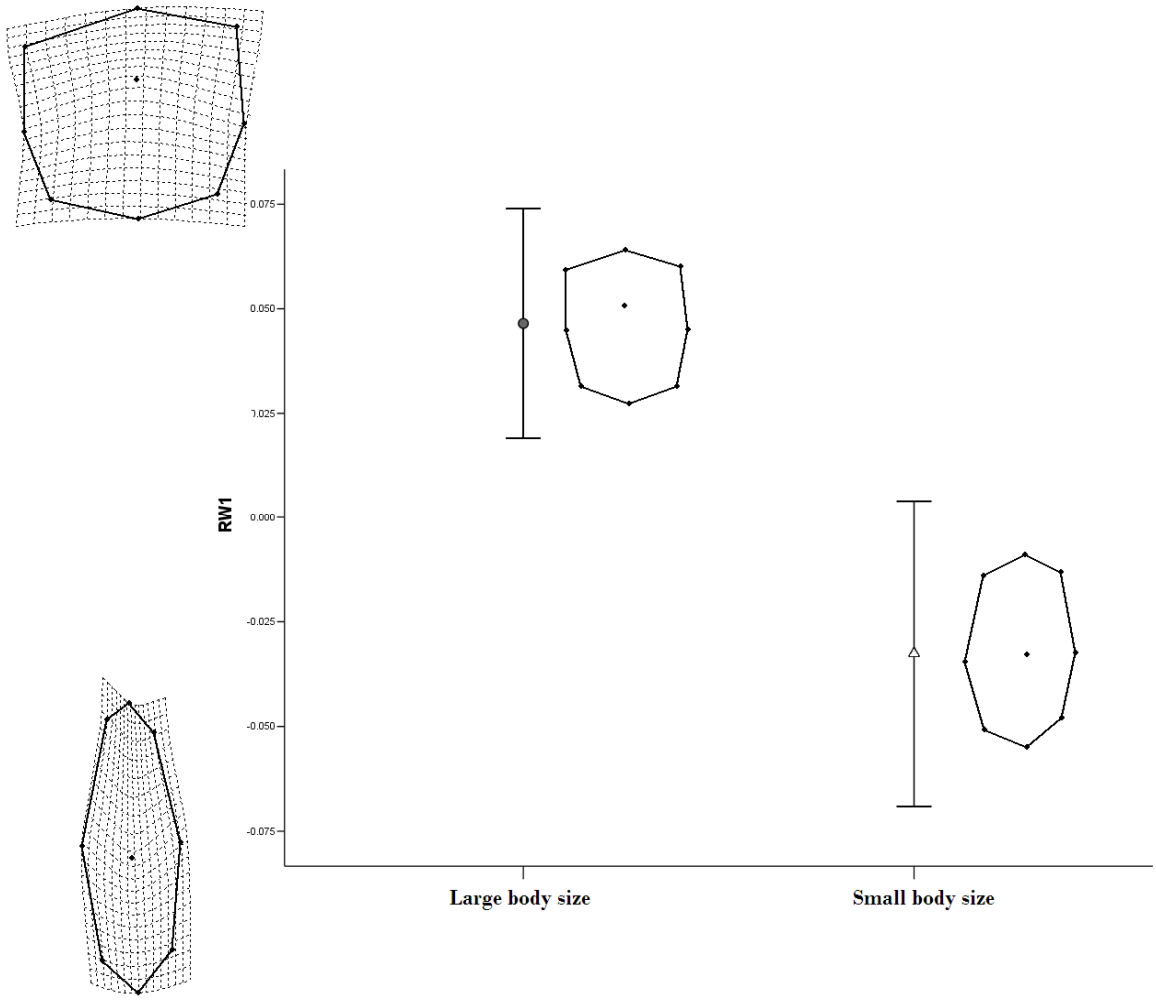

Fig. 6.2 Relative Warp 1 scores mean ±S.E. indicating the shapes of the more extreme values for the axis. Splines of the actual mean shape for small body size and large body size phenotypes from Loch Tay are depicted.

Overall, the shape of scales from the autumn and spring spawning phenotypes of S. alpinus from Loch Awe also showed significantly differences (Goodall's F-test F14, 392= 2.84; P = 0.0004). The RW1 explained 46% of the total variation in shape of scales. RW1 scores differed significantly between the sympatric phenotypes from Loch Awe (F1,28= 5.2; P = 0.03). The consensus shape of the scales of the two phenotypes is shown in Fig. 6.3. The spring spawning phenotype had scales that are elongated and thin; whereas the autumn spawners had more rounded and laterally expanded scales. The scales also vary significantly in centroid size (F1, 28= 6.88; P = 0.014), spring spawners had smaller scales (1.42±0.07 mm) than autumn spawners (1.78±0.1mm).

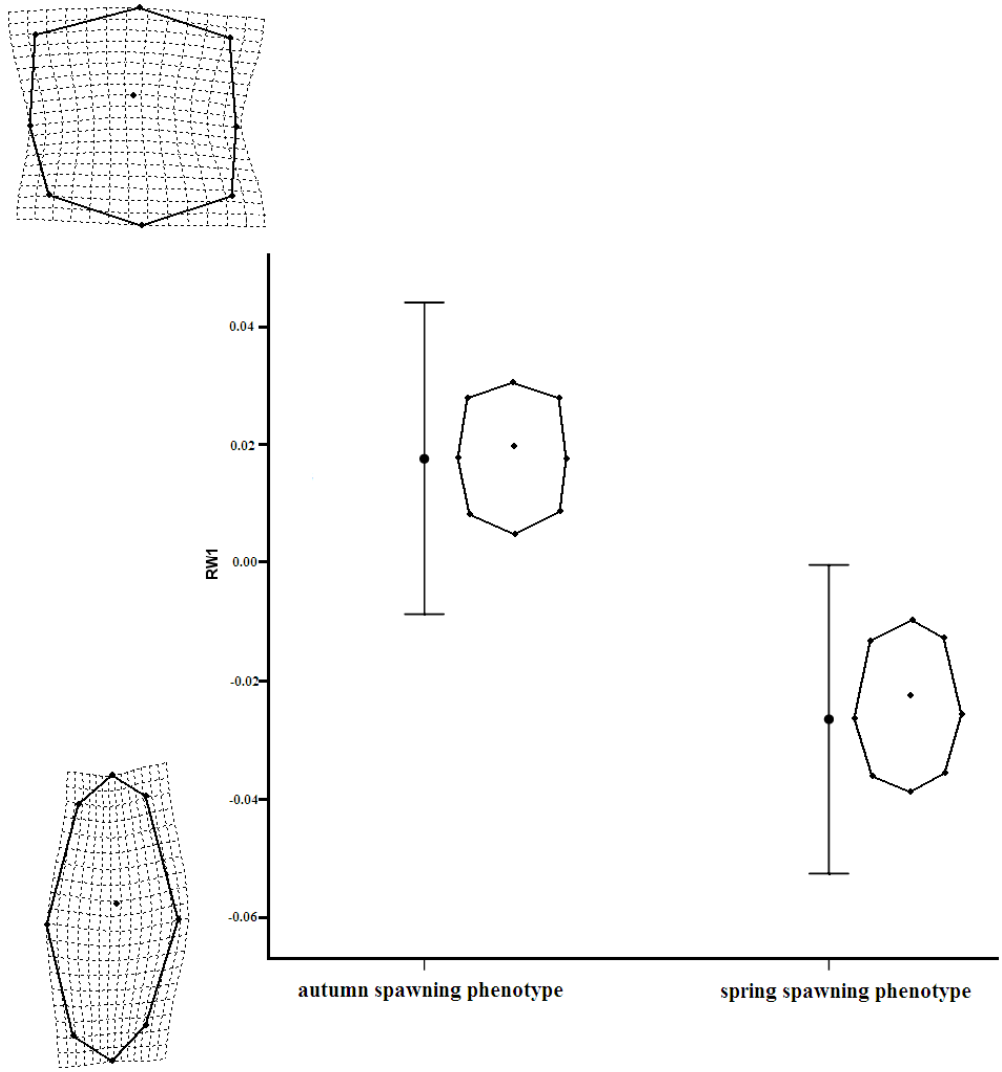

Fig. 6.3 Relative Warp 1 scores mean ±S.E. indicating the shapes of the more extreme values for the axis. Splines of the actual mean shape for autumn spawning and spring spawning phenotypes from Loch Awe are depicted.

6.4. Discussion

Here it is shown that scale shape, analysed with the geometric morphometric technique, has the ability to discriminate between closely related phenotypes of the same species living in sympatry. Scales morphology may represent an important phenotypic characteristic for fish as they interact with the surrounding environment through their scales and have a potential influence in swimming performance (Long et al., 1996). The size of scales appears to be functionally significant to fish, for example small scales provide greater protection to internal organs and muscles and provide more hydrodynamism (Sudo et al., 2002). Therefore, the variation in scale shape between closely

related alternative phenotypes of the same species described here may thus reflect known ecological and life history differences between forms. In Loch Awe the spawning phenotypes show dietary segregation, the spring spawning phenotype feed on macro-benthic prey whereas the autumn spawning phenotype feed on plankton, they also differ in trophic and body morphology that corresponds to diet. The alternative phenotypes from Loch Tay also show significant differences in diet and morphology, the small-body size phenotype is a littoral zone inhabitant whereas the large-body size phenotype feed in the pelagic zone. Furthermore, in both lochs the alternative phenotypes exhibit difference in the growth rate (see chapter 5).

In conclusion, the use of Geometric morphometric methods applied to fish scales can provide a useful tool to discriminate among sympatric alternative phenotypes. Moreover, it is suggested as an important complementary tool that could be use as a first screening for the presence of such phenotypes. Its use also could help to clarify the integrity of species in some individual populations and very important it can be use as a quick, non destructive, inexpensive and informative technique as suggested by Ibanez et al. (2009).

CHAPTER 7. GENERAL DISCUSSION

In this thesis five important studies addressed the role of coexisting expressed alternative phenotypes within a single species in the route to full speciation, using the hypothetical framework steps of incipient speciation suggested by West-Eberhard, 2003.

In the first step of the model the expression of alternative phenotypes within a single species is required to initiate variation upon which selection can act. The expression of alternative phenotypes is often thought to be the result of ontogenetic processes and specifically phenotypic plasticity responses to exposure to different environmental conditions. In fish, which have been widely used to test such questions, exposure to different diets is the most frequently described initiator of plastic responses; this is supported in the first half of chapter 4. However, less attention has been paid to the effect of physical environment. In chapter 2 it was shown that the exposure of the three-spined sticklebacks to different habitats resulted in expression of very significant differences in body and head morphologies and spine position, demonstrating that physical environment can modulate the expression of traits through phenotypic plasticity during ontogeny.

It is now well-known that the effect of diet is of major importance (Bertrand *et al.*, 2008; Michaud *et al.*, 2008; Amundsen *et al.*, 2008; Wund *et al.*, 2008; Malaquias *et al.*, 2009) and that specialisation in alternative prey items leads to significant variation in trophic morphology, as demonstrated in chapter 3. Thus, habitat characteristics together with the presence of different prey may represent one route to morphological variation but also each, habitat and diet, separately represent environmental inputs that trigger phenotypic divergence and the establishment of divergent, discrete, or bimodally distributed complex alternative phenotypes. Therefore, these two environmental inputs can affect a whole population in one generation. This process can thus spread and increase, in frequency, the expression of the novel phenotypes.

When morphological differences arise, discrete morphological characteristics may be originated and reinforced by the continuous presence of same environmental conditions (i.e. same alternative prey and/or same alternative habitats), then individuals specialise on specific prey or habitat types. Thus alternative phenotypic expressions may become fixed in the population.

Many animal species show individual foraging specialisms where potential prey require prey-specific foraging strategies (Uchii *et al.*, 2007). Arctic charr are often found as benthic (macroinvertebrates) or pelagic (plankton) foraging specialists. Here, we tested specifically if, given a choice of prey with different characteristics, individuals would specialise and if individuals would chose prey based on their expressed trophic morphology. When offered benthic and pelagic prey items most individuals (73%) showed 100% fidelity to a single foraging source. Naïve individuals (not previously exposed to natural prey) with more robust head and mouth shape were more likely to forage on a benthic prey source (chironomids). In contrast, individuals with a more fusiform body, larger eye but more slender head shape were more likely to specialise on pelagic prey (*Artemia*). These results support the hypotheses that the availability of discretely different prey types can result in the degree of foraging specialisms which may result in discrete alternative phenotypic expressions through subsequent plastic ontogenetic processes. Then this expression of alternative phenotypes will be maintained by selection pressures.

Ecological specialisation may lead to extremely rapid evolutionary divergence of populations in different habitats and may be an important mechanism leading to rapid ecological speciation, that occurs because of selection and adaptation to different environments (Schluter, 1995; Nagel & Schluter, 1998).

Morphological variation driven by plasticity is suggested to be linked with parameters that promote genetic isolation (West-Eberhard, 1989; Day & McPhail, 1996). The most likely intrinsic isolating mechanism is arguably assortative mating. This mechanism is commonly suggested as an isolating mechanism between closely related species (Scott, 2004; Ólafsdóttir *et al.*, 2006; Hendry *et al.*, 2009). The crucial issue is the extent to which character under divergent selection also promotes assortative mating. The results presented in chapter 4 showed clear evidence of assortative mating on the basis of the diet-induce body morphology. Females chose to mate with morphologically similar males, depending on their own morphology. Thus, the preference of the female for a specific trait (i.e. shape) increases directional selection on specific male phenotypes (Kokko *et al.*, 2007). This study thus demonstrated that morphological variation by means of plasticity represent one potential sexual selection route through which gene pool segregation may occur. Therefore phenotypic plasticity by means of assortative mating can influence speciation rates in sympatry.

Here, the behaviour showed by the three-spined sticklebacks females (mate choice) may be labile, thus the assortative mating demonstrated may be incidental more than adaptive. Consequently, assortative mating between individuals of "like phenotype" may provoke an incidental accumulation of morph-specific genetic divergence in alleles that affect regulation and form (see step 3 of West-Eberhard model). Although, female choice is considered relevant to increase the genetic quality of offspring by choice of ecologically compatible mates that express a parallel phenotype (see step 4 of West-Eberhard model)

In natural populations however, coexisting phenotypes can either originate by intralacustrine divergence of one founder population (sympatry) or by multiple invasions of the forms representing different lineages (Robinson *et al.*, 2000a). In chapter 5, contrasting sympatric alternative-phenotype systems from two Scottish lakes were shown. Loch Tay charr exhibit a bimodal size-frequency distribution amongst sexually mature fish whereas Loch Awe charr are unimodal in body size, but segregate into two distinct spawning phenotypes.

The results of this study demonstrate that Arctic charr in both lakes show clear evidence of trophic segregation between forms and to a large extent these phenotypes are ecologically segregated. In each lake the stable isotope values and morphology are indicative of a planktonic foraging phenotype and a littoral foraging phenotype. In both systems there is evidence that the two forms are operating as separate gene pools.

It is suggested that the Loch Awe forms segregated very recently and most likely while in sympatry. Although, the parameters that directed the initial segregation of spawning phenotypes are unknown, assortative mating may be implied, due to the segregation in the spawning time and the clear ecological segregation demonstrated. Thus a rapid evolutionary divergence of the population using different foraging habitats may be leading to a mutual acceleration of bidirectional divergence (phenotypic and genetic) in regulation and form, which may be further accelerated by character release and bidirectional sexual selection (step 5 of West-Eberhard model). Then the presence of phenotypes ecologically and morphologically segregated is leading to the fixation of the single alternatives (step 6 of the West-Eberhard model) which due to difference in spawning time are also reproductively isolated (step 7). The small genetic distances between different spawning phenotypes are expected because extensively reorganized new phenotypes can occur with little genetic change (West-Eberhard, 2005a).

The alternative phenotype hypothesis also applies to the large body-size and the small body-sized phenotypes from Loch Tay. Although there are no direct data to support this, the increased phenotypic divergence between them may lead to the forms exhibiting strong assortative mating. This is reflected by the significant genotypic divergence, that also represents effective reproductive isolation, and the fact that both phenotypes spawn in the same place at the same time. However, here it is suggested that this segregation was directed not by sympatric divergence by itself but by the ecological adaptation of the forms in allopatry. These alternative phenotypes represent a more ancient segregation potentially (but not certainly) pre-post glacial invasion.

In summary, the divergent developmental pathways within species enable the exploitation of different conditions and resources by individuals of the same species as adaptive options, and assortative mating by developmentally similar individuals then contribute to speciation. The evolution of a divergent novelty does not require gene-pool divergence, only developmental-pathway and gene-expression divergence. Phenotypic differences that ultimately distinguish species arise before the initiation of reproductive isolation between them, because the origin and maintenance of more than one developmental pathway can occur within a population (West-Eberhard, 2005b). Therefore, some phenotypic divergence assumed to mark species may in fact represent intraspecific alternative phenotypes representing gene-expression differences and not genetic, differences between individuals, such may be the case of the Arctic charr complex in British Isles (Adams *et al.*, 2006; Adams & Maitland, 2007).

Here, it is shown that pre-isolation divergence by means of developmental plasticity can make an enormous contribution to the evolution of reproductive isolation and genepool segregation, supporting the hypothesis of ecological speciation, which seeks to associate pre-isolation divergence under selection with the origin of reproductive isolation, whether in sympatry or allopatry. Finally, although not all divergence is via alternative phenotypes, these maybe ranked along with speciation as an important panorama in evolutionary divergence.

7.1. Future work

More phenotype environmental input-induced research must be carried out. The effect of habitat, diet and other environmental inputs should be followed through generations within a species (e.g. Three-spined sticklebacks). Genetic work must be used not to describe the results of speciation but to describe the factors that cause it. Genomic studies must contemplate from now gene-expression. Research on patterns of gene expression makes it possible to pinpoint the (expressed) loci that are actually subject to selection in the evolution of species differences, beginning with differences that arise because of developmental recombination without reproductive isolation (West-Eberhard, 2005b).

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