Morphological and behavioural responses of threespine stickleback (*Gasterosteus aculeatus*) to abrupt alterations in their selective landscape

by

Stephen Leaver
BSc, University of Victoria, 2007

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Colonization events provide a tremendous opportunity to examine ecological associations and evolutionary processes, but researchers rarely have the opportunity to study the original colonizers, and document the progression of changes that occur in response to the new environment. In 1993 T.E. Reimchen transferred 100 threespine stickleback (*Gasterosteus aculeatus*) between fundamentally different habitats on the island archipelago of Haida Gwaii, British Columbia. The source, Mayer Lake, is a well studied, morphologically conserved, population of uniquely giant and darkly pigmented population inhabiting a large, dystrophic, darkly stained, and vertebrate predator (primarily cutthroat trout (*Oncorhynchus clarki*) and diving birds such as the common loon (*Gavia immer*)) dominated habitat. The transplant locality, Mayer Pond, is a small, eutrophic, and invertebrate predator (mainly dragonfly larvae of the genus *Aeshna*) dominated, and previously fish free pond.

Fourteen years later, using digital photographic measurements of wild-caught specimens and lab-raised individuals I examined the presence, extent, and rate of morphological changes in the transplanted population. Analysis of approximately 20 morphological traits on 272 wild-caught (collected from Mayer Lake in 1929 and 2003, and Mayer Pond in 1994, 1995, 1998, 2003, 2007, and 2009) and 105 lab-raised fish (eggs collected in 2007) demonstrate an immediate (present in the first generation following transplant) and persistent reduction of gill raker length, dorsal and pelvic spine length, and the gradual, progressive decline in presence of

anterior lateral plates in the transplant population. I interpret these morphological differences in light of the growing interest surrounding phenotypic plasticity and the potential for genetic assimilation.

Using the lab-raised individuals I also conducted a diversity of simple preference trials and predatory stimuli experiments to evaluate differences in the antipredator behaviour of fish from the two habitats. Although there were no indications of predator-specific differences between the two populations, fish from the low predation transplant habitat, Mayer Pond, are consistently more active in a novel environment and recover more quickly following a stressful event than fish from the higher predation pressure source population, Mayer Lake. I evaluate these behavioural differences between the populations with respect to predator sensitive foraging theory.

Threespine sticklebacks have colonized a wide diversity of habitats since the end of the last glaciation approximately 11,000 years ago. Although their adaptability is clear, my results provide evidence of rapid evolutionary change in heritable aspects of morphology and behaviour following only 14 years in a novel habitat, as well as previously undocumented levels of morphological plasticity, both of which likely contribute to the widespread success of this species.

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Dedication

This thesis is dedicated to those who seek knowledge and understanding of the fascinating world around us.

Chapter 1: General Introduction

There is a growing interest in the potential for rapid evolutionary change (Ford 1964, reviews in Hendry and Kinnison 1999, Darimont *et al.* 2009). Although Darwin recognized that natural selection was constantly operating, he expected that it was primarily through the "long lapse of the ages" (Darwin 1860, page 84) that we could see evolutionary change. However, there is evidence that evolutionary change can occur within just a few generations in natural populations (Cain and Sheppard 1954, Ford 1964, reviews in Reznick *et al.* 1997), and even seasonal changes in selective pressures are reflected in frequency changes of phenotypes within a population (Reimchen 1995). Additionally, environmentally induced phenotypic plasticity can alter an individual's phenotype in response to various selective pressures (West-Eberhard 2005). Although plasticity has conventionally been associated with low heritability, and therefore not typically considered as influenced by the mechanism of natural selection, plasticity potentially has a genetic basis and can therefore be acted upon by natural selection (Gotthard and Nylin 1995). Furthermore, plasticity may act as a 'first step' in evolution by natural selection by driving subsequent heritable changes in phenotype (Price *et al.* 2003, West-Eberhard 2005). One of the best ways to study both the speed and the mechanisms (change in heritable phenotypes by natural selection, or phenotypic plasticity) of the evolutionary process, as well as gain further insight into the ecological role of various traits, is through colonization events where there is an abrupt change in the selective landscape faced by a population (Schluter 1996b).

Some of the best examples of landscape level changes are caused by the movement of glaciers during episodic ice ages (Hewitt 1996). During the last ice age glaciers covered much of the northern hemisphere, and when they receded novel terrestrial and aquatic habitat became available to colonizers from glacial refugia. The threespine stickleback (*Gasterosteus aculeatus*) was one such colonizer and has become a model for evolutionary studies investigating the resulting phenotypic changes that occur in response to these new environments (Wootton 1976, 1984).

Threespine sticklebacks have become a model species for a number of reasons (Bell and Foster 1994, Bell 1995a):

1) They are geographically widespread (circumpolar distribution including North America, Europe, Asia, and Japan) and environmentally diverse (found in marine, estuarine, and freshwater environments) which allows for comparisons across ecological conditions and extensive geographic separation.
2) Much of their current freshwater habitat was only exposed following the last glaciation. Therefore, many current populations are the result of independent colonizations of these newly available habitats by a common marine ancestor within a known time span.
3) They are morphologically diverse. Instead of scales armoured plates occur along the side of the fish, and the number and size of these plates varies between populations. Additionally, articulated and lockable spines replace the first dorsal and pelvic fin. The dimensions of these defensive features, as well as feeding structures such as gill raker length, eye diameter, and jaw length, vary between localities and ecological conditions. Even body shape has been shown to reflect different ecological conditions (Spoljaric and Reimchen 2007).
4) They are behaviourally complex. They have elabourate courtship displays and nesting behaviour (first notably studied by Tinbergen 1952) and show differences in behaviour between individuals and populations.
5) They are not only common in the wild, but are relatively small (maximum 100 millimeters) and can be reared and bred in the lab. Hybrids between phenotypically extreme individuals between populations are also often viable allowing for detailed examination of the genetic mechanisms controlling phenotype.
6) Due to the scientific interest generated by the above five points, much of their genome has been sequenced (Peichel *et al.* 2001) allowing for even greater insight into the genetic fine tuning of evolutionary changes.

One location where there has been much work examining the adaptive significance of variation using threespine stickleback is on Haida Gwaii, an island archipelago off the West coast of British Columbia(reviewed in Wootton 2009). Sticklebacks colonized lakes on Haida Gwaii following the end of the last ice age approximately 12,000 years ago (Moodie and Reimchen 1976a, Reimchen and Nosil 2006), as they did in many other parts of the world (Reusch *et al.* 2001, reviewed in Wootton 2009). What makes Haida Gwaii unique, however, is the tremendous diversity among lakes in ecological conditions including abiotic factors such as

water clarity and chemistry, and biotic features such as predation pressure (Reimchen 1994). The independent colonization by marine stickleback of these freshwater environments across a number of replicated ecological gradients allows for thorough investigations into the relationships between selective pressures and resulting phenotypic variation (Reimchen and Nosil 2006).

Predation is an important component of the selective landscape. The consequences of not reacting to predation are grave (Lima and Dill 1990), and predation pressure shapes all aspects of phenotype including physiology, life history, behaviour, and morphology (Endler 1995). Stickleback predators on Haida Gwaii are diverse. Reimchen (1994) lists over 60 potential predators of stickleback, with piscivorous fish such as cutthroat trout (*Oncorhynchus clarki*), and diving birds such as the common loon (*Gavia immer*), being the most important sources of mortality at one lake in Haida Gwaii.

Variation in predators between localities results in predictable correlations with phenotype. For example, gape limited, toothed, trout predators favour an increase in spine length to maximize a fish's effective diameter (Hoogland *et al.* 1956), as well as an increased number of anterior plates to protect against puncture wounds (Reimchen 1992). On the other extreme, invertebrate predators, such as dragonfly larvae of the genus *Aeshna*, grab hold of stickleback in ambush, and in populations where these are the dominant source of mortality, fish often have reduced or absent spines and armour to reduce the grappling ability of this predator (Reimchen 1980, Marchinko 2009).

Ecological context can also modify the predation sequence and the resulting optimal phenotype. For example, in locations where there are fast-moving trout predators, and waters are clear and relatively vegetation-free, stickleback are likely to be caught when pursued and therefore have robust post-capture defences such as a greater amount of body armour (Reimchen 1994). If trout are replaced by diving birds (which cannot sustain an underwater pursuit for as long) fish tend to have less armour (Reimchen 1994), a tradeoff of post-capture defense for increased burst acceleration and a higher probability of escape during pursuit (Bergstrom 2002). The repeated and predictable correlation between the forms of predation, ecological conditions, and resulting phenotype of residing stickleback supports the hypothesis that predation is an important selective force modifying phenotype.

Although the relationship between form and function has been well documented, these populations have potentially had thousands of years to develop these associations. How quickly

did the first populations adapt to the change in selective landscape offered by these new habitats compared to the marine environment? Did the correlations we observe today take much of the time since the recession of the glaciers, or did populations change quickly and persist in the adaptive form? These questions tie into a broader attempt to understand the rates and mechanisms of evolution.

To further investigate the relationship between selective landscape and phenotype, as well as the rates and mechanisms of changes that result in this relationship, T.E. Reimchen carried out a simple common garden experiment. In 1993 he transplanted 100 adult sticklebacks from one well studied lake on Haida Gwaii to a fundamentally different ecological environment. The source of the transplant, Mayer Lake, is a large, darkly stained, body of water. Previous work has identified Mayer Lake stickleback as a unique form of gigantic, darkly pigmented 'Black stickleback' that have had a conserved body form over many years (Moodie 1972). These fish reach reproductive size (~75 mm) in three years and females are capable of producing more than one clutch per season (Moodie 1972). T.E. Reimchen took these unusual and highly conserved fish and transferred them into a nearby small, turbid water, water body he called 'Mayer Pond'. In addition to limnological differences between these habitats, Mayer Lake has diving birds (such as common loons) and piscivorous fish (primarily cutthroat trout) as the primary sources of mortality, while Mayer Pond lacks these vertebrate predators (the water body is isolated and never colonized by fish predators, and it is too small for the diving bird predators seen in Mayer Lake) and instead only has invertebrate predators such as *Aeshna* (dragonfly) larvae (T.E. Reimchen, personal communication). Additionally, based on habitat, one would expect Mayer Lake fish to have a more planktonic diet, while Mayer Pond sticklebacks have a more benthic prey field. Accordingly, T.E. Reimchen had a number of predictions about the resulting direction of morphological change. He expected there to be a shift from a streamlined to a deep body, from many to fewer gill rakers, and a shift towards fewer lateral plates due to the different limnological features, prey field, and predator characteristics of the new habitat (T.E. Reimchen, personal communication).

Fourteen years later I began trying to identify any morphological and behavioral changes in the transplanted population using multiple wild-caught collections and lab-raised specimens from the source and transplant locality. My primary focus was to identify if any changes have taken place. Secondarily, if there are changes, what has been the direction and rate of change? Were changes slow and progressive over multiple generations, or were features modified

immediately in the new environmental conditions? A progressive shift in the phenotype of the wild-caught collections over successive years since the original transplant corresponds to expectations of heritable changes caused by natural selection. (An inference further supported if these features are also detectably different in fish from the two populations raised entirely in the lab.) Conversely, if the full extent of change occurs in the first generations of transplanted individuals, and there are no detectable differences in lab-raised individuals, then this would provide evidence for phenotypic plasticity in the feature. As I do not have behavioural comparisons from wild-caught Mayer Lake and Mayer Pond fish, I can only look for the presence of any inherited differences in lab-raised fish after 14 years since separation from the original population, not the rate at which these changes occurred.

Chapter 2: Materials and Methods

2.1 Study locality and transplant details

I compared two sites representing fundamentally different habitats located on the Northeast corner of the Haida Gwaii archipelago on the West coast of Canada (Table 1 and Figure 1). Mayer Lake is a large, darkly stained, vertebrate-dominated lake colonized by threespine stickleback from a marine ancestor sometime after the glaciers receded approximately 11,000 years ago. Mayer Pond is a small, turbid watered and vertebrate predator reduced environment created with the paving of Highway 16 between Tllel and Port Clements around 50 years ago (T.E. Reimchen, personal communication). It was fish free until 1993 when T.E. Reimchen transferred 100 adult sticklebacks from Mayer Lake to Mayer Pond. The current population estimate for Mayer Pond based on the size of the water body and sampling effort is approximately 5000 fish (T.E. Reimchen, personal communication). As a general estimate, the population size of stickleback in Mayer Lake is thought to number in the hundreds of thousands (Moodie 1984).

Table 1: Comparison of abiotic and biotic features of Mayer Lake and Mayer Pond

	Mayer Lake (Moodie 1972, 1984)	Mayer Pond (T.E. Reimchen, personal communication)
Location	53.68° N, -132.05° W	53.628° N, -132.044° W
Size	627 ha	0.03 ha
Max depth	9m	1.5m
Mean depth	2.6m	1m
Water clarity	Stained	Turbid
Macrophytes	Uncommon, some littoral (approximately 20% of shoreline)	Extensive
Primary predators	cutthroat trout, diving birds (i.e. Common loons), conspecifics	Invertebrates (particularly *Aeshna* larvae), conspecifics

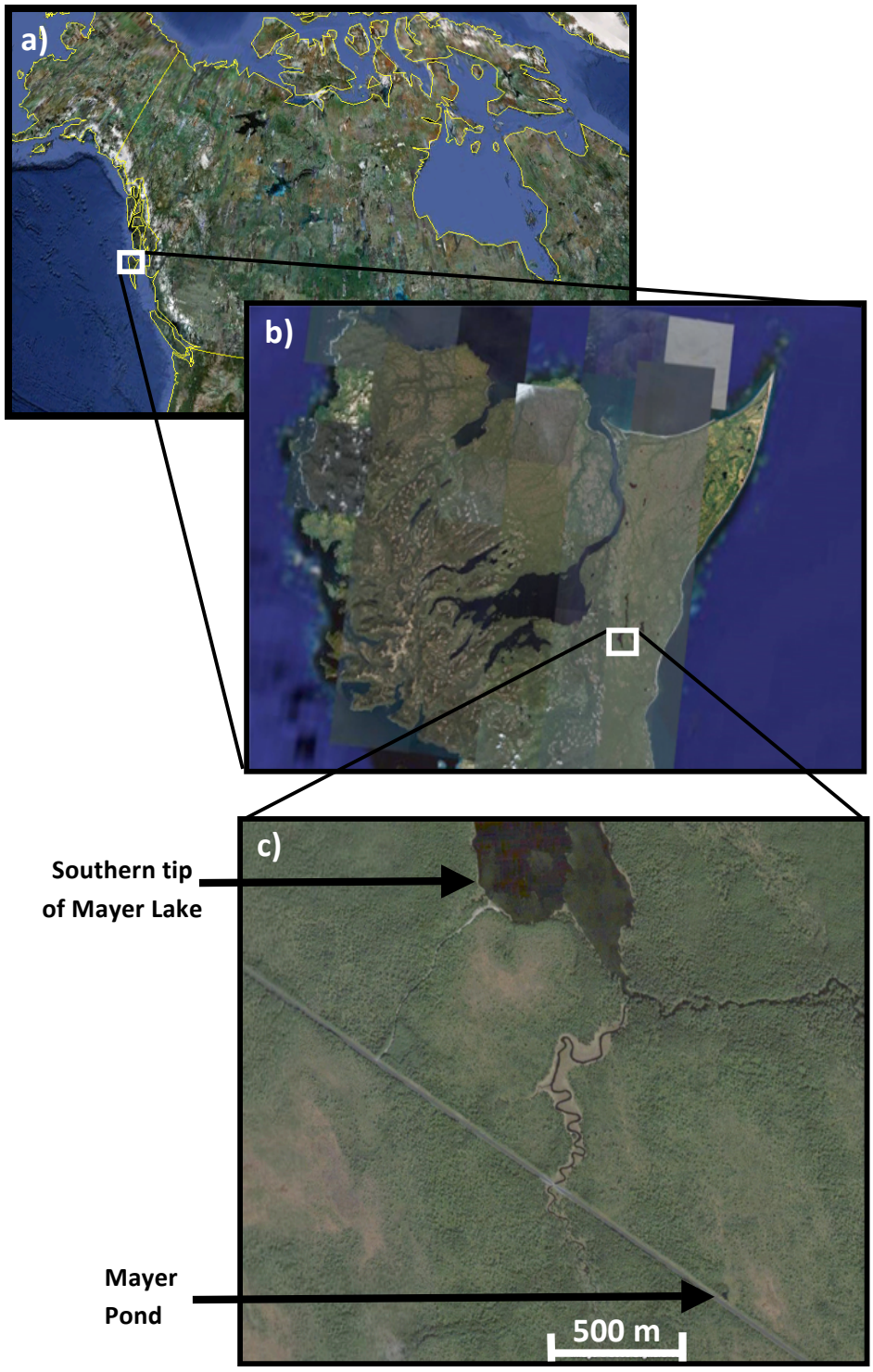


Figure 1: The island archipelago of Haida Gwaii is located on the west coast of British Columbia, Canada (a). The two study sites, located on the northeast corner of Graham Island (b), include Mayer Lake and the nearby Mayer Pond (c). Scale bar on (c) represents 500 meters. *Source:* "Mayer Lake and Mayer Pond." 53.634°N and -132.048°W. Google Earth. Image compiled August 8, 2005. Image accessed October 29, 2008.

2.2 Wild-caught collections for morphological comparison

All samples, other than the 1929 Mayer Lake sample which was collected using a beach seine, were collected by T.E. Reimchen using 8 millimeter (mm) diagonal mesh minnow traps baited with old cheddar cheese and left overnight in less than 2 meters (m) of water. Collected fish were euthanized and stored in ethanol. The 1929 Mayer Lake sample was first preserved in formalin. I examined fish collected from Mayer Lake in 1929 and 2003, and from Mayer Pond in 1994, 1995, 1998, 2003, 2007, and 2009.

2.3 Dietary comparison of Mayer Lake and Mayer Pond

To evaluate differences in the potential prey fields of Mayer Lake and Mayer Pond, I conducted a dietary analysis of the stomach contents of 63 similarly sized (50-60 mm) wild-caught specimens (25 individuals from two Mayer Lake samples, and 38 individuals from three Mayer Pond collections). These collections occurred over a number of years, but were seasonally similar (late May to early September). For each specimen I removed the entire digestive tract and examined the contents under a dissecting scope. I distinguished three different orders of organisms: Diptera (primarily family Chironomidae), Cladocera, and Ostracoda. Dipterans were present in both Mayer Lake and Mayer Pond, but cladocerans were only found in Mayer Lake, and ostracods were only found in one Mayer Pond sample (see Table 2, Figure 2, Figure 3).

Table 2: Mean percent of the total number of items within a stomach that belong to one of three orders of prey organisms for two Mayer Lake and three Mayer Pond samples.

		Dipteran	Ostracod	Cladoceran
Mayer Lake	1929	55%	0%	45%
Mayer Lake	2003	2%	0%	98%
Mayer Pond	1994	100%	0%	0%
Mayer Pond	1997	100%	0%	0%
Mayer Pond	2003	48%	52%	0%

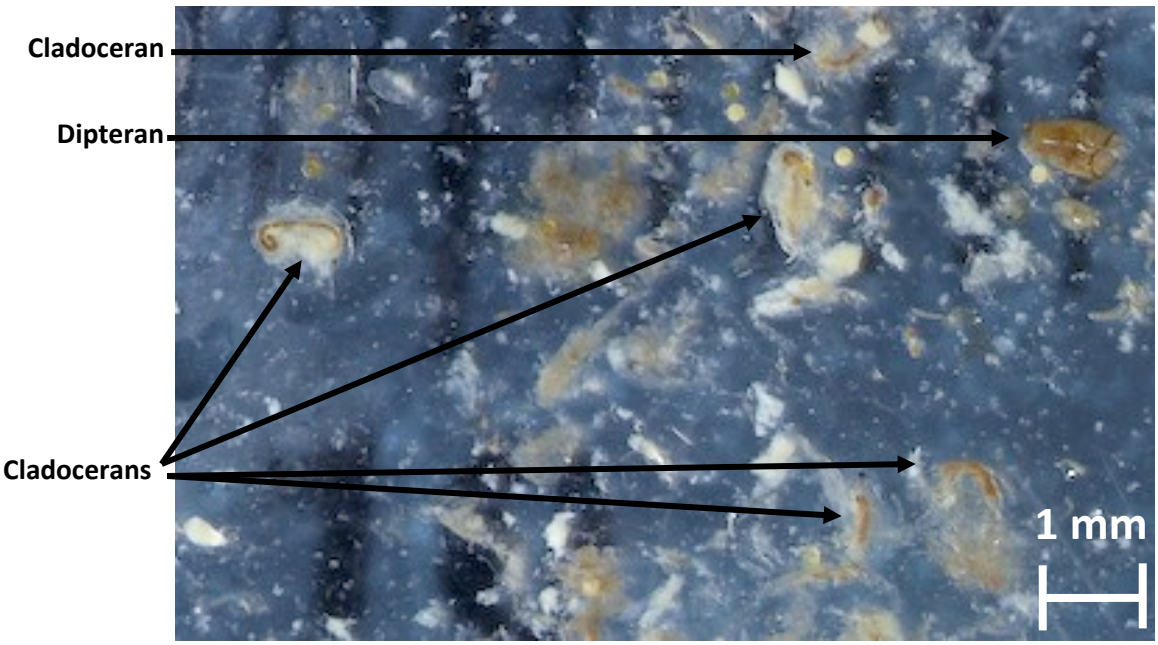


Figure 2: Mayer Lake stomach content example. Typical of fish collected from Mayer Lake there are Dipterans and Cladocerans present. Scale bar represents one mm.

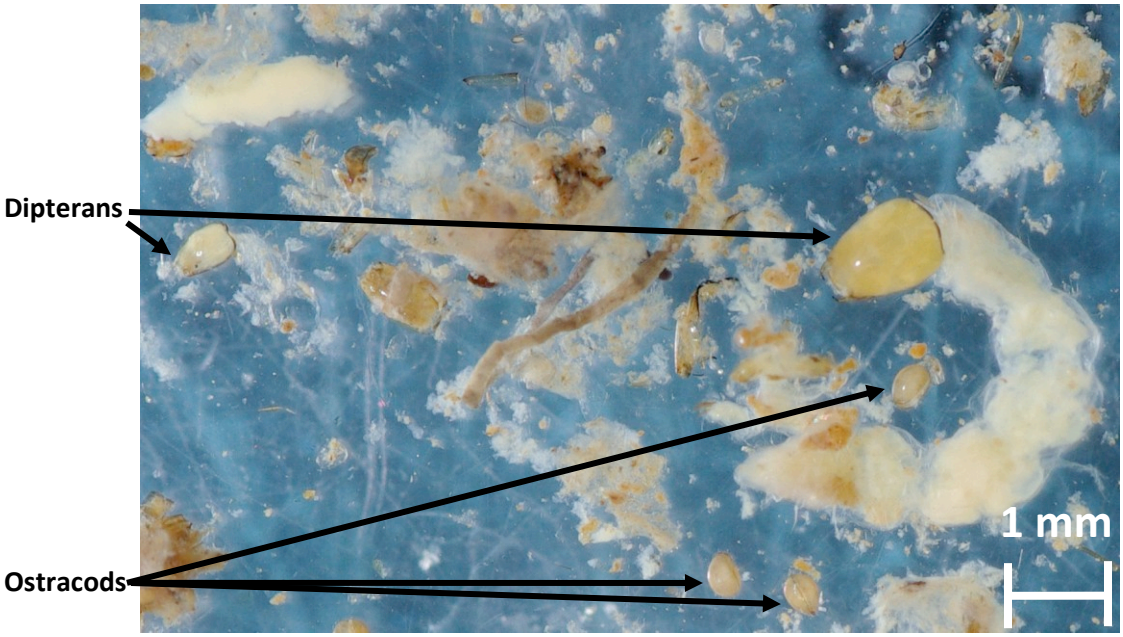


Figure 3: Mayer Pond stomach contents example. Dipterans and Ostracods are present. Scale bar represents one mm.

There was some variation in the stomach contents between the samples, but this may be due to collecting technique. For example, the Mayer Lake 1929 sample had the greatest number of identifiable prey items compared to all other samples, and a much more equal representation of dipterans and cladocerans than the Mayer Lake 2003 sample (Table 3). However, this was the only sample collected by beach seine. The rest of the samples were collected using minnow traps left overnight. There was a noticeable degradation in the number and quality of identifiable prey items in the minnow trap samples versus the beach seine collection, likely due to the increased time for food items to be eliminated from the digestive tract, or just more extensively digested, in fish that had been in traps potentially for hours.

Table 3: Average counts of items (max and min in brackets) from three different orders (dipterans, ostracods, and cladocerans) of prey items in two samples from Mayer Lake and three samples from Mayer Pond. The sample size and proportion of samples with no identifiable contents is listed as well.

Lake	Year	Sample size	Proportion of sample with no identifiable contents	Mean dipteran count (min, max)	Mean ostracod count (min, max)	Mean cladoceran count (min, max)
Mayer Lake	1929	15	0.07	12.8 (2, 30)	0	14 (4, 30)
Mayer Lake	2003	10	0.3	1.5 (1, 2)	0	17.6 (8, 26)
Mayer Pond	1994	15	0.53	3 (1, 8)	0	0
Mayer Pond	1997	13	0.15	5.5 (1, 21)	0	0
Mayer Pond	2003	10	0.3	3 (1, 7)	10 (5, 24)	0

The differences in prey availability between Mayer Lake and Mayer Pond relate to expected ecological differences between the two habitat types. Mayer Lake is a large, open, body of water and stickleback primarily feed in the limnetic zone (Moodie 1984). Mayer Pond lacks this open water limnetic zone - individuals are restricted to benthic zone foraging. Schluter and McPhail (1992) describe the prey items likely to be found in limnetic and benthic zones. Cladocerans are found in limnetic areas, ostracods occur in benthic habitats, and dipteran chironomids occur in both (Schluter and McPhail 1992). The prey items I identified match the expected habitats of Mayer Lake and Mayer Pond. As differences between benthic and limnetic feeding have resulted in morphological divergence, and potentially speciation, of stickleback even within the same lake (for review see Taylor and McPhail 1999), these differences in prey

between Mayer Lake and Mayer Pond provide the potential for evolutionary changes in feeding structures.

2.4 Lab-raised fish collection and rearing for behavioural and morphological comparisons

I collected fertilized eggs from nests in Mayer Lake and Mayer Pond in July 2007 and reared the resulting offspring in the controlled laboratory conditions of the University of Victoria Aquatic Facility. Nests of fertilized eggs were first identified by fanning and nest care behaviour by male sticklebacks, and then collected by hand. I removed the egg masses from the nest and washed them with an antifungal methylene blue solution before suspending the fertilized eggs by mesh in aerated buckets of lake water. For transport, the eggs were placed in 50 milliliter (mL) centrifuge tubes filled almost completely with aerated lake water, chilled to 5° Celsius, and packed with ice packs and Styrofoam in a cooler. Upon arrival at UVic each clutch of eggs was transferred into an individually screened container suspended in aerated, common system water (deliberately buffered to have a pH of 5.5, approximately halfway between the pH of Mayer Lake and Mayer Pond).

In total I collected five clutches of eggs from Mayer Lake, and three clutches of eggs from Mayer Pond. However, two of the three Mayer Pond clutches were lost to fungus before the eggs hatched, and due to pH fluctuations in the system water approximately four months into rearing a further 90% of fish from each clutch died (Table 4). To eliminate this physiological stress the pH of system water was gradually raised to a neutral pH of 7 around four months into rearing and mortalities subsequently stopped.

Table 4: Number of hatched fry from Mayer Lake and Mayer Pond and percent mortality after 6 months

Clutch	Number of fry hatched	Number surviving after 6 months	Percent mortality
Mayer Lake 1	280	11	96.1%
Mayer Lake 2	130	28	78.5%
Mayer Lake 3	85	33	61.2%
Mayer Lake 4	197	23	88.3%
Mayer Lake 5	78	7	91.0%
Mayer Pond	317	32	89.9%

All clutches were maintained in equivalent conditions throughout rearing. As the fish grew they were progressively transferred from small screened containers, to five gallon rat cages, to divided 45 gallon tanks. The clutches were kept in the same system water, but in separate partitions. The Mayer Pond clutch was also separated into two equal size groups soon after hatching due to the large size of that clutch, and to potentially examine environmental differences between supposedly equivalent rearing environments. Fish were subject to a seasonal and natural daylight cycle (fluorescent lights turned on and off by a light sensor on the roof). Newly hatched fry were fed brine shrimp nauplii two to three times daily, ad libitum. Once the fish were large enough I primarily fed them bloodworms, but also occasionally 0.2 mm trout pellets and frozen krill for nutritional variety. Once behavioural experiments were complete and fish were large enough to compare with wild-caught counterparts, I euthanized the fish with an overdose of Tricainemethanesulfonate (MS-222 or TMS), according to the University of Victoria Aquatic Unit facility Standard Operating Procedure OA2003 for euthanizing fish, and preserved the specimens in 70% ethanol for further analysis.

Chapter 3: Morphological changes in wild-caught stickleback following transplantation of fish from Mayer Lake to Mayer Pond

3.1 Introduction

The defensive morphology of the threespine stickleback is quite unique, and has received considerable attention by evolutionary biologists (for review see Wootton 2009). This species has armoured bony plates instead of scales, and spines that replace some of the rays of the dorsal and pelvic fins. The pelvic girdle is also fused into a bony ventral plate. Combined with a bony ascending process arising from the pelvic plate, lateral plates on the sides of the fish, and bony supports below the dorsal spines, this ventral pelvic plate completes a protective structure which not only encompasses the fish, but supports the erectable dorsal and pelvic spines which can puncture the mouths of predators (see Figure 4 and Reimchen 1983) as well as simply increasing the fishes' effective diameter (Hoogland *et al.* 1956).

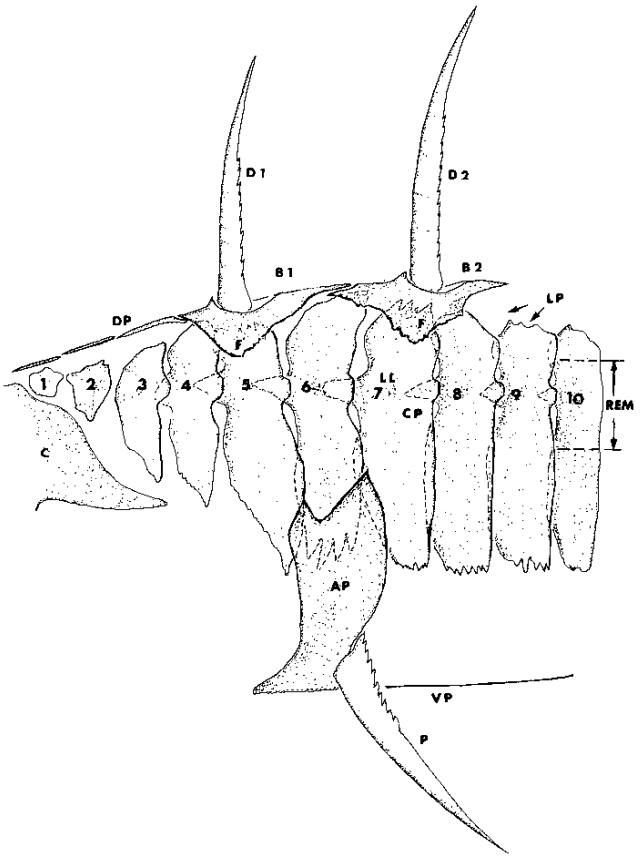


Figure 4: Lateral image of threespine stickleback illustrating several defensive structures (reproduced with permission from Reimchen, 1983). D= dorsal spine, P=pelvic spine, VP=ventral plate, AP=Ascending process, LP=Lateral plate (individual plate positions numbered 1 - 10).

Additionally, variability in the defensive morphology of this species has predictable correlations with different types of predation pressure and ecological contexts. The ancestral marine stickleback is almost always fully plated, meaning that bony lateral plates run the length of the fish, one on each myomere. This morphology is also seen in very clear freshwater environments (Reimchen 2000). Fish predators are often present in both habitats. In these situations, selective pressures have favoured post-capture armour as a defense against puncturing toothed predators which are unlikely to lose sight of their stickleback prey in clear water, and which have no requirements for air forcing them to abandon pursuit (Reimchen 2000). In contrast, in habitats where the water is darkly stained, vegetation is more prevalent, or predators are more likely to be diving birds that have a limited pursuit time before they must come up for air (and have no teeth to puncture a sticklebacks skin), a reduction in armour is favoured (Reimchen 1995) to improve burst acceleration (Bergstrom 2002) and capitalize on evading the predator during the pursuit phase of predation, rather than escaping after capture. The presence of macro-invertebrate predators such as dragonfly larvae, which capture stickleback through a grappling ambush, can further the encourage reduction of armour and spines, and even result in the complete loss of these features in some populations, so as to improve the chance of escape after being grabbed (Reimchen 1980).

With specific respect to lateral plates, different plate positions have particular antipredator roles. Anterior plates (positions 1-3) protect against puncturing by toothed fish predators (Reimchen 1992). Plates positioned mid-body (positions 4-8) buttress dorsal and pelvic spines and resist compression and deflection of those spines when captured by a predator (Reimchen 1983). Posterior plates (positions 9-30) do not support spines, but interfere with the swallowing abilities of piscivores, possibly by interfering with the action of the predator's pharyngeal jaws, and thereby improve the chances of escape (Reimchen 2000).

Less conspicuous, but also well studied, is the relationship between feeding structure morphology and ecological context in threespine stickleback (Lavin and McPhail 1987). The importance of feeding differences, and the resulting morphological divergence, is best exemplified in stickleback by the extensive work on benthic-limnetic species pairs that occur in a number of lakes (McPhail 1984). In these habitats, spatial differences between two subgroups of stickleback – one primarily benthic, the other mainly limnetic – has led to morphological divergences with benthic individuals being deeper bodied, with larger eyes, shorter jaws, and

fewer, shorter gill rakers (Peichel *et al.* 2001). These differences, thought to be caused primarily by different diets (Schluter and McPhail 1992), are sufficient to result in assortative mating within each group and there is potentially sufficient divergence between the pairs that some consider them separate species (Taylor and McPhail 1999).

Given the demonstrated relationship between morphological variability and ecological context in threespine stickleback, one can make a number of predictions as to the direction of morphological change in Mayer Pond fish from the Mayer Lake ancestors. First, with respect to defensive features, Mayer Lake predators are predominantly cutthroat trout and diving birds. Both are gape limited, and selection should therefore favour stickleback to have longer spines to avoid being swallowed. Cutthroat trout also result in an increased prevalence of anterior lateral plates in sticklebacks to prevent puncture wounds caused by this predator (Reimchen 1992). Mayer Pond not only lacks these predators, and thus doesn't have this selective direction for longer spines and anterior armour plates, but the primary predators in this environment are dragonfly larvae, which exert an opposite selective pressure and encourage the reduction of armour and spines (Reimchen 1980). Additionally, given that armour is energetically costly (Giles 1983b) and potentially unnecessary, I predict that shorter spines, fewer lateral plates, and reduction in all bony defensive features (e.g. ascending process, pelvic plate) will be selected for in Mayer Pond. With regard to feeding structures, both the habitat and prey field (see Chapter 2) of Mayer Pond more closely resemble that described as benthic by other authors (Schluter and McPhail 1992), while Mayer Lake is more limnetic (Moodie 1972). Therefore, I expect Mayer Pond fish to exhibit a more 'benthic' feeding morphology than the potentially 'limnetic' form of Mayer Lake individuals, manifesting with a deeper body, larger eyes, shorter jaws, and fewer, shorter, gill rakers in Mayer Pond fish.

As well as investigating whether these changes occur, I am interested in what rate, and by what mechanism, changes take place. By examining samples taken over successive generations since the original transplant, I identify whether changes are the result of progressive changes in the distribution of phenotypes over time, potentially indicating changes brought about by selective pressures and the differential fitness of different morphologies, or if any changes occur immediately and persist – possibly indicative of an environmentally induced, phenotypically plastic, response.

In addition to simply looking at each morphological feature in samples from both populations, I will also examine the sexual dimorphism in each trait, as well as the asymmetry

between left and right sides for features present and measured on both sides of the fish. Sticklebacks have considerable morphological sexual dimorphism which may be related to different ecological niches of the sexes (Reimchen and Nosil 2004, Reimchen *et al.* 2008, Spoljaric and Reimchen 2008). The extent and direction of asymmetry, particularly with regard to lateral plate presence, has also been found to reflect ecological niche differences (Reimchen *et al.* 2008). I am interested in seeing whether these two additional morphological indices will be altered by the abrupt change in selective landscape caused by the transplant of fish to Mayer Pond from Mayer Lake.

3.2 Materials and Methods

3.2.1 Measurements/repeatability

Approximately 30 measurements were taken from each specimen. I measured fish in hand for standard length and maximum depth (depth immediately anterior to the first dorsal spine) using vernier calipers (accurate to 0.1 mm), and used a dissecting scope to obtain counts and position of left and right lateral plates (according to landmarks detailed in Reimchen 1983) as well as to sex the fish. The first gill arch on the left side was removed and stained with an alizarin red solution for 20 to 40 minutes before being left overnight in 95% ethanol. I used a 10 megapixel camera (Pentax K10d) with a macro lens (Pentax 100mm f2.8) and an accessory flash (Sigma EF – 500 DG Super) to take detailed photos from: the left and right sides of the fish, the underside of the fish, the front of the fish (specifically showing the width of the first spine) and the extracted/stained gill arch which had been pressed into one plane using a slide and coverslip. The same set of vernier calipers provided an equivalent scale in all photos. Using this scale and ImageJ software (Rasband 2009) I was able to obtain measurements representing feeding structures (left eye width and height, left jaw length, gill raker number, gill raker length and gill raker spacing) as well as defense structures (the width and height of non-overlapping left and right lateral plates - typically positions one, two, and three - when present, the length and width of the first dorsal spine, the length of the second dorsal spine, the length of the pelvic spine, the length and area of the pelvic plate, and the height and total area of the ascending process connecting the pelvic process to the lateral plates). Figure 5 through Figure 8 show example photographs with these measurements overlaid.

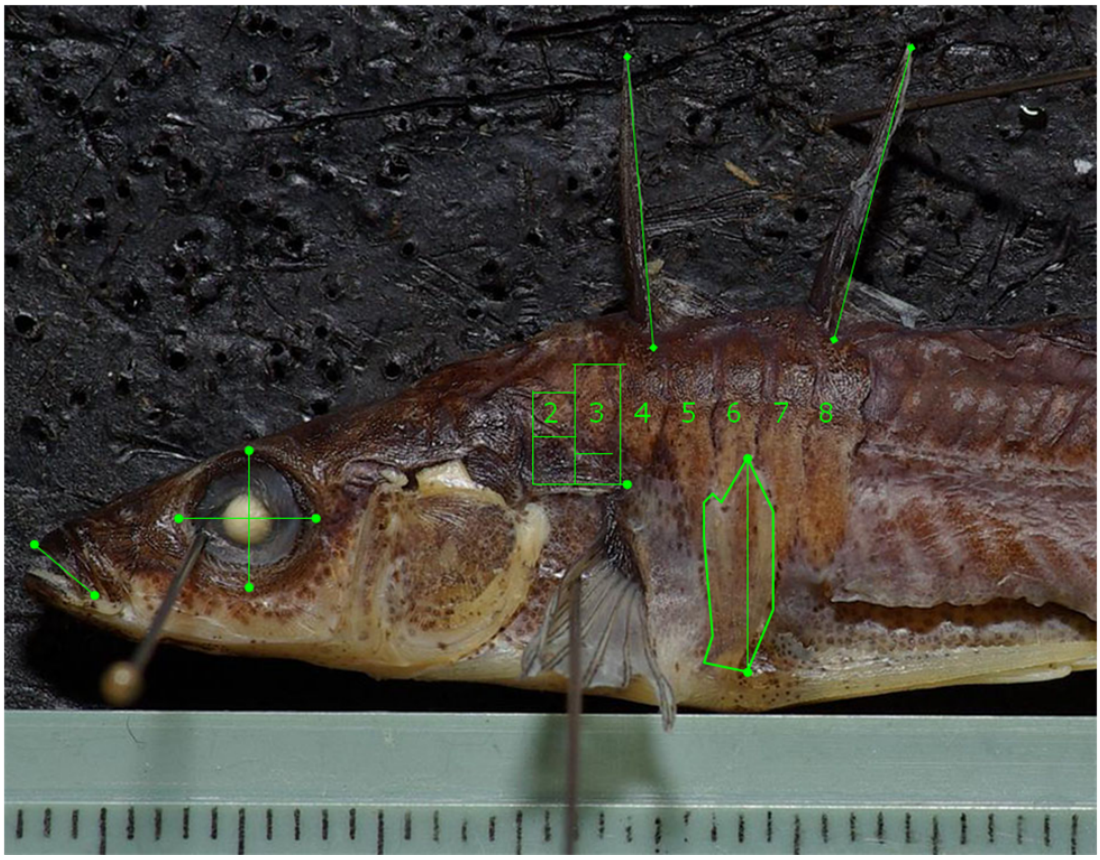


Figure 5: Example photograph from the left side of the fish. Measurements taken are marked in green and include: Left jaw length, left eye width, left eye height, plate dimensions (width and height) of plate positions 1, 2, and 3 when present, the length of the first and second dorsal spine, and the height and area of the ascending process. These features were used to evaluate the extent and rate of morphological change of a transplanted population from its source population after 16 years of separation. Each unit on the scale bar below represents one millimeter.

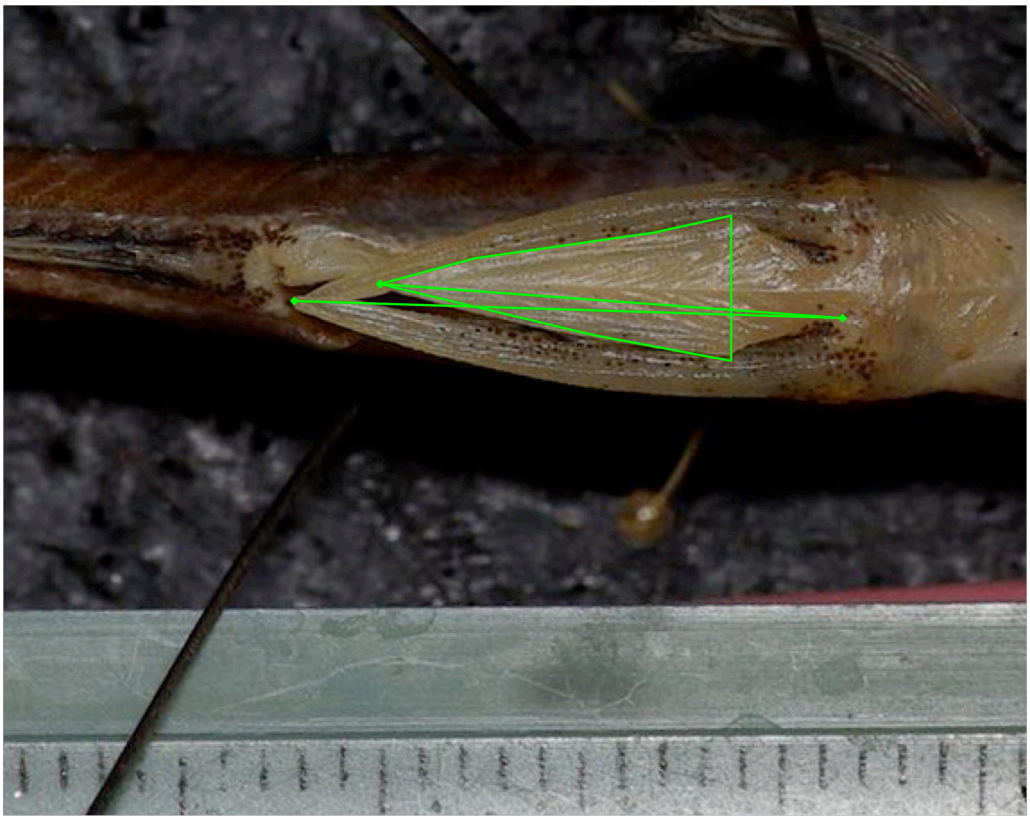


Figure 6: Example photograph of the underside of a specimen. Measurements shown in green include the length of the pelvic plate and left pelvic spine (as measured from the point where the pelvic spine and plate meet), as well as the area of the pelvic plate (defined by the three outside points of the pelvic plate). Each unit on the scale bar below represents one millimeter.

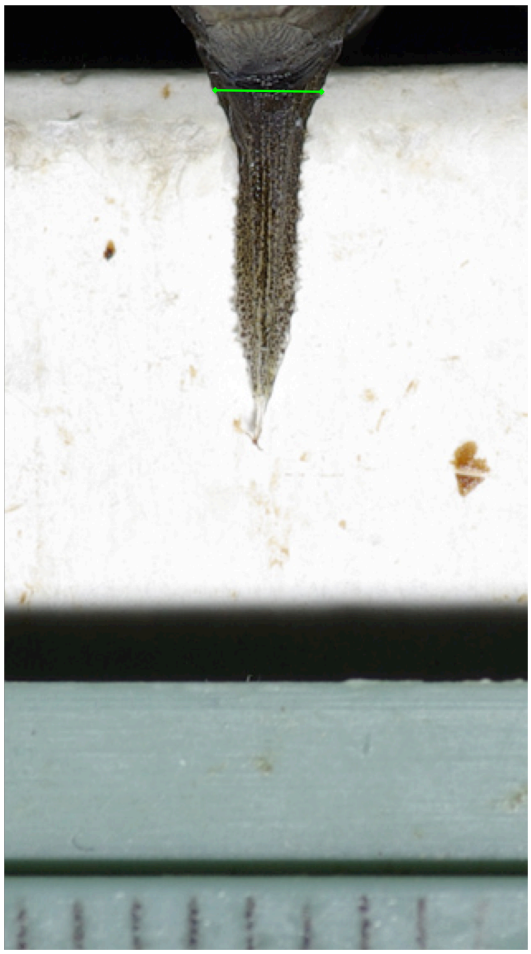


Figure 7: Example photograph taken from the front of the fish highlighting the width of the first spine. The measurement (shown in green) was the width, perpendicular to the long axis of the spine, at the most distal ridge marks that occurred across the base of the spine. Each unit on the scale bar below represents one millimeter.

Figure 8: Example photograph of an extracted first left gill arch that has been stained with alizarin red, placed in 95% ethanol, and then photographed when flattened between a coverslip and a slide. Measurements highlighted with green include the count of gill rakers on the upper and lower branch of the gill arch, the length of calcified and stained portion of the first, third, and fifth gill raker below the joint between the upper and lower branch, as well as the spacing between them. Each unit on the scale bar below represents one millimeter.

To assess the repeatability of morphological measurements I randomly selected 16 wild-caught fish (eight each from Mayer Lake and Mayer Pond) and re-measured all morphological traits. The average difference between the measurements for all traits was 2.7%, with the least repeatable being the right ascending process area measurement at 11.2% error (Table 5).

As many of these measurements are highly correlated (e.g. left eye width and height), instead of reporting two fairly equivalent sets of results from highly correlated traits, I used these values of precision to choose the trait with the least measurement error (e.g. left eye height) to analyze in depth, and report the correlation of the second measurement (e.g. left eye width) as well as note any deviations in pattern if the correlation is low. Therefore, of the duplicated measurements, the eye measurement analyzed is left eye height, the dorsal spine value is from the second dorsal spine, plate dimensions are represented by lateral plate height (a lower error than width when both are averaged across plates 2 and 3 as well as the left and right sides), and gill raker length is illustrated by gill raker 5 bone length.

Table 5: Average percent difference between two measurements taken from the same fish. Eight Mayer Lake and eight Mayer Pond fish were randomly re-measured to evaluate the repeatability of 23 different morphological features. The average percent difference between measurements for all morphological features was 2.7%. Bolded traits are those analyzed in depth in the morphological analysis of Chapter 3, chosen either because they are a unique trait, or because they are the most precise measurement given a choice of highly correlated options (e.g. Left eye height is the most precise, and therefore the best, proxy of eye dimension).

Measurement	Mayer Lake	Mayer Pond	Average across lakes
Left eye width	1.39%	0.79%	1.09%
Left eye height	**0.53%**	**0.26%**	**0.40%**
Left jaw length	**2.25%**	**2.88%**	**2.56%**
First spine length	1.02%	0.94%	0.98%
Second spine length	**0.24%**	**0.58%**	**0.41%**
Left lateral plate 2 width	3.77%	3.31%	3.54%
Left lateral plate 2 height	**5.80%**	**2.74%**	**4.27%**
Left lateral plate 3 width	5.52%	1.69%	3.60%
Left lateral plate 3 height	**0.63%**	**2.82%**	**1.73%**
Left ascending process area	**1.49%**	**5.95%**	**3.72%**
Right lateral plate 2 width	5.35%	3.24%	4.29%
Right lateral plate 2 height	**1.52%**	**1.46%**	**1.49%**
Right lateral plate 3 width	0.91%	5.21%	3.06%
Right lateral plate 3 height	**4.80%**	**5.04%**	**4.92%**
Right ascending process area	**5.18%**	**17.25%**	**11.22%**
Left pelvic spine length	**1.55%**	**1.77%**	**1.66%**
Pelvic plate area	**0.20%**	**0.06%**	**0.13%**
First spine width	**0.20%**	**6.28%**	**3.24%**
Gill raker 3 length	3.01%	0.49%	1.75%
Gill raker 5 length	1.23%	6.23%	3.73%
Gill raker 1 to 5 distance	**1.40%**	**0.77%**	**1.08%**
Gill raker 3 bone length	0.30%	0.75%	0.53%
Gill raker 5 bone length	**0.43%**	**0.04%**	**0.23%**

3.2.2 Data analysis

The morphological data have two forms - continuous data as in found in most of the morphological features, and meristic data such as the number of lateral plates and gill rakers - and each required a different form of statistical analysis. For the continuous data, I used an ANOVA with Tukey's post-hoc comparisons to identify differences between Mayer Lake and each Mayer Pond sample for each feature. The count data analysis consisted primarily of comparing the proportion of a sample that had a specific lateral plate; in this case I used a general linear model with a binomial error structure to interpret changes in the proportion data over successive generations. For the other count data, total gill rakers, I used a general linear model with a Poisson error structure to determine if there were differences in the number of gill rakers over time since separation of Mayer Pond from Mayer Lake.

Before I could analyze the continuous data, I had to compensate for potential differences in the size of each fish as these morphological features are expected to be significantly correlated with standard length. To allow comparisons irrespective of standard length, I standardized each datum to that as would be found in a 60 mm fish (the average size of fish in the transplant population). To do this I took the residuals obtained by regressing the feature of interest (e.g. left eye height) with standard length for each sex and sample separately. Then, as residuals from larger fish cannot be considered equivalent to those of the same magnitude from smaller fish (as equal values for a smaller fish represent a proportionally bigger feature – e.g. a 10mm spine on a 50mm fish should be considered proportionally larger than a 10mm spine on an 80mm fish) I multiplied each residual by the ratio of 60/standard length of that fish. I combined these proportionally appropriate residuals with the expected value of a 60mm fish as determined by the regression of that sample and sex. This effectively maintained the variability in the data, while clustering points around the mean value of that sample and sex for a 60mm fish, and allowed comparisons between samples and sexes without further compensation for differences due to standard length. Count data, such as for plate or gill raker numbers, were not included in this process as the literature indicates that the gill raker number and total plate number are established by 28 mm standard length (Hagen 1973) and are not likely to be influenced by ontogenetic or size related changes in fish between 40mm and 95mm.

As Mayer Lake has an unchanging stickleback population (Moodie 1972), and I found no statistical differences between the 1929 and 2003 samples in any of the traits, I compared

Mayer Pond to the Mayer Lake 1929 and 2003 samples pooled together. Since the Mayer Lake 2003 sample is most representative (in both time and sampling location) of the collection which founded the Mayer Pond population, the addition of the Mayer Lake 1929 sample provides a more conservative baseline from which to measure any deviation in the Mayer Pond fish. (Note: due to previous destructive sampling, no data was obtained from the Mayer Lake 1929 samples for eye dimension, jaw length, or first spine width.)

For asymmetry, I subtracted the left value from the right value (right-left) for each fish that had the feature present on at least one side. The resulting data were compared similar to the other features, with an ANOVA and Tukey Post hoc analysis to identify if there were any differences between samples.

As well, since I expected sexual dimorphism in these traits (Spoljaric and Reimchen, 2008), I conducted the analyses for males and females separately. To examine the degree of sexual dimorphism over time since separation, I subtracted the mean female value from the mean male value (male-female) within a sample for each trait. As the resulting values have no variance, I qualitatively looked for trends and differences between the populations rather than conducting statistical analyses.

Due to the likely covariance between many of the morphological features, I first conducted a multivariate principal components analysis to identify the combination of traits that best explains the variation in the data, and investigated if these principal components were different between Mayer Lake and Mayer Pond. I used SPSS version 14 to generate a principal component analysis using the corrected values of the following features: first, second, and dorsal spine length, lateral plate three dimensions, ascending process dimensions, pelvic plate length, and gill raker length and spacing.

Since the principal component analysis can only be done on a subset of the measured individuals and features (principal components are only generated for individuals that have a value for every feature and, as not all fish have all features – e.g. left lateral plate 3, there is an inherent compromise between the number of features and the sample size of fish included in the analysis) I also examined each morphological feature individually. To prevent repetition from reporting similar results from highly correlated individual features, I restricted my results to the most precise measurements (see end of section 3.2.1 regarding measurement error) of a given trait (e.g. eye size, dorsal spine length), and note the correlation (and deviation in trend if present) of similar measurements. Using SPSS version 14 and R version 2.9.2 (Team 2009) I

examined these individual morphological features to identify if there have been any changes in Mayer Pond fish compared to Mayer Lake individuals, and, if so, at what rate these changes occurred over successive years since the original transplant. To provide an ecological context to any changes, I grouped the analyses of these individual features into functional sets as follows:

1) Feeding structures – body depth, eye dimensions, jaw length, gill raker number, and gill raker length and spacing.
2) Defensive structures – proportion of fish with protective lateral plates, the size of these lateral plates, the dimensions of the dorsal and pelvic spines, and the size of the pelvic plate and ascending process which buttress those spines.

3.3 Results

3.3.1 Sample summary

I examined a total of 272 fish (74 from Mayer Lake, 198 from Mayer Pond; Table 6).

Table 6: Numbers of male and female threespine stickleback examined from eight different samples to identify the extent and rate of morphological changes that may occur when an organism's selective landscape is drastically altered. The two Mayer Lake samples (ML) represent the source population for comparison and span a long 74 year time frame to provide insight into the consistency of features in the original population. The six Mayer Pond (MP) samples start with samples taken shortly after the original 1993 transplant, and continue until the most recent 2009 sample. Values in brackets represent the mean standard length (in millimeters) of the fish belonging to that particular sample and sex.

	ML 1929	ML 2003	MP 1994	MP 1995	MP 1998	MP 2003	MP 2007	MP 2009
Male	20 (67.0)	28 (66.1)	12 (56.8)	14 (58.5)	18 (53.9)	15 (56.6)	15 (57.0)	20 (53.2)
Female	9 (69.0)	17 (64.9)	9 (57.0)	7 (62.3)	16 (55.9)	15 (56.6)	32 (66.2)	25 (66.9)
Total	**29**	**45**	**21**	**21**	**34**	**30**	**47**	**45**

3.3.2 Multivariate assessment

The multivariate principal component analysis included 237 individuals (25 from ML29, 37 from ML03, 20 from MP94, 21 from MP95, 27 from MP98, 30 from MP03, 36 from MP07, 41 from MP09). The first three principal components (PC) explained 36.5%, 13.1%, and 12.5% of the variance, respectively. PC1 is primarily a reflection of longer spine and gill rakers, PC2 of larger lateral plate 3 dimensions, and longer pelvic spines and pelvic plates, and PC3 of taller ascending processes (Table 7).

Table 7: Principal component (PC) loading breakdown. Main contributing features are bolded.

Feature	Principal component		
	1	2	3
First spine length	**0.80**	0.16	-0.02
Second spine length	**0.71**	0.28	0.00
Left lateral plate 3 width	0.31	**0.48**	0.08
Left lateral plate 3 height	0.65	**0.44**	0.14
Left ascending process area	0.57	-0.33	0.43
Left ascending process height	0.26	-0.41	**0.71**
Right lateral plate 3 width	0.36	**0.50**	0.02
Right lateral plate 3 height	0.62	0.35	0.11
Right ascending process area	0.61	-0.32	0.29
Right ascending process height	0.37	-0.35	**0.69**
Left pelvic spine length	0.65	**0.42**	0.05
Pelvic plate length	0.23	**0.49**	0.51
Gill raker 3 length	**0.82**	-0.28	-0.35
Gill raker 3 bone length	**0.80**	-0.28	-0.39
Gill raker 5 length	**0.82**	-0.30	-0.31
Gill raker 5 bone length	**0.82**	-0.31	-0.37
Gill raker 1 to 5 distance	-0.09	-0.24	0.28

Of the three principal components generated, PC 1 is the best at discriminating Mayer Lake and Mayer Pond (Figure 9). As Mayer Lake fish have higher values than Mayer Pond specimens, we can infer that fish in the transplant population have shorter dorsal spines and gill rakers compared to the source population. The other two principal components not only explain considerably less variation than PC1, but do not discriminate between Mayer Lake and Mayer Pond and were therefore not considered further.

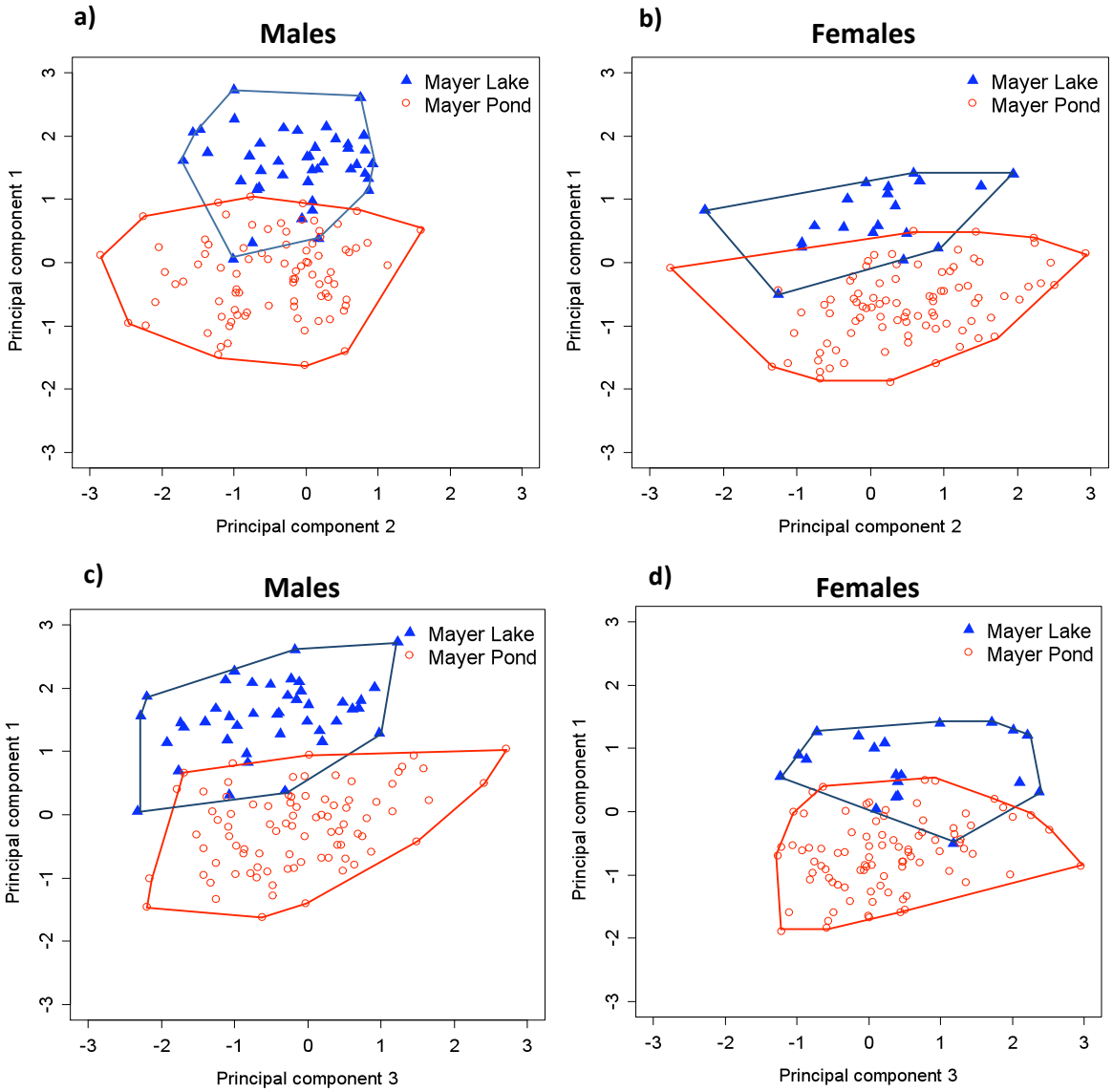


Figure 9: Scatterplots of three principal components (PC) comparing Mayer Lake (solid, triangles) and Mayer Pond (open, circles) to identify which combination of traits best identifies differences between the source and transplant population. The perimeter points are bounded to show the maximal range in both axes of each principal component, as well as the degree of overlap between principal components. PC1 is plotted against PC 2 for (a) males and (b) females, and against PC3 for (c) males and (d) females. Mayer Lake and Mayer Pond separate most along the Principal component 1 axis, with Mayer Lake having higher values than Mayer Pond, and therefore indicating that Mayer Lake fish primarily have longer dorsal spines and gill rakers than Mayer Pond specimens.

The full extent of change in PC1 between Mayer Lake and Mayer Pond fish is realized in the first generations and persists in all samples (Figure 10). This trend is replicated in males and females, with a significant reduction in Mayer Pond compared to Mayer Lake for both sexes and all samples (Tukey Post hoc comparison of each Mayer Pond sample to Mayer Lake, all $p<0.001$).

Males always have larger values than females, but the degree of sexual dimorphism fluctuates, with the MP03 sample having the greatest dimorphism, and the most recent MP09 sample displaying less dimorphism than the original Mayer Lake population (Figure 10).

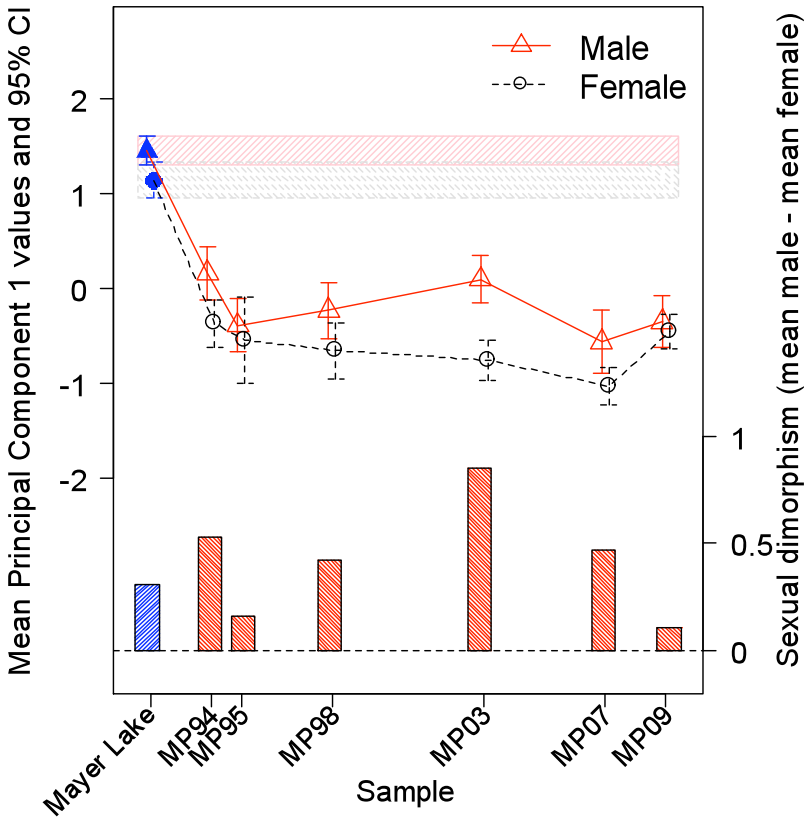


Figure 10: Mean Principal component 1 values and 95% confidence intervals for males (triangles, solid line) and females (circles, dotted line) from Mayer Lake (solid symbols) and subsequent samples from Mayer Pond (open symbols). Mayer Pond collections are placed along the x-axis on a scale appropriate to their separation in time. The 95% confidence intervals of the source population Mayer Lake are extended across the graph with a solid lined, right sloped hatching rectangle for males, and a dotted lined, left sloped hatching rectangle for females. The bar graph at the base is described by the axis on the right hand side, and represents the degree of sexual dimorphism within each sample (mean male value – mean female value) for the Mayer Lake (left slope lined, bar) and the six Mayer Pond samples (right slope lined bars).

3.3.3 Individual feeding structures

Body depth

The length-corrected values of body depth initially decrease in Mayer Pond fish, but then increase over successive samples. The 1995 and 1998 Mayer Pond samples in males, and the 1994, 1995 and 1998 samples in females, all have significantly thinner depths compared to Mayer Lake fish (Tukey post-hoc comparisons, all $p<0.03$). There is an increase in body depth in subsequent years and the most recent sample from Mayer Pond in 2009 contains males and females with significantly deeper bodies than the baseline from Mayer Lake ($p<0.001$ for males and females; Figure 11). The sexual dimorphism appears to initially increase, but decline to almost no difference between males and females in the most recent samples from Mayer Pond (Figure 11).

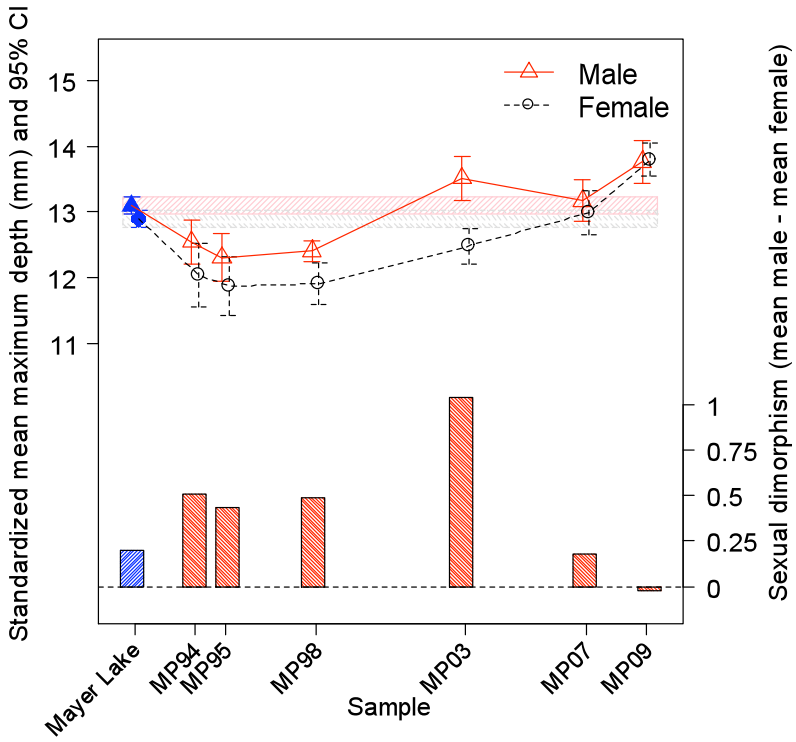


Figure 11: Standardized mean maximum depth in millimeters with 95% confidence intervals as well as bar graph illustrating extent of sexual dimorphism. All maximum depth values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Left eye size

There is an increase in the height of the left eye in Mayer Pond fish compared to Mayer Lake samples. This difference occurs in all male samples (Tukey post hoc test, all $p<0.001$). In females, there is no difference in the first two samples (Tukey post-hoc comparison with ML $p=0.07$ for MP94 and $p=0.16$ for MP95) but the rest of the samples are significantly larger than Mayer Lake females (all other $p<0.01$; Figure 12). The width of the left eye is highly correlated with the height measurements (Pearson Correlation =0.6, $p<0.001$) and shows similar trends.

The degree of sexual dimorphism appears to be staying the same (Figure 12), but in all samples males have larger eyes than females.

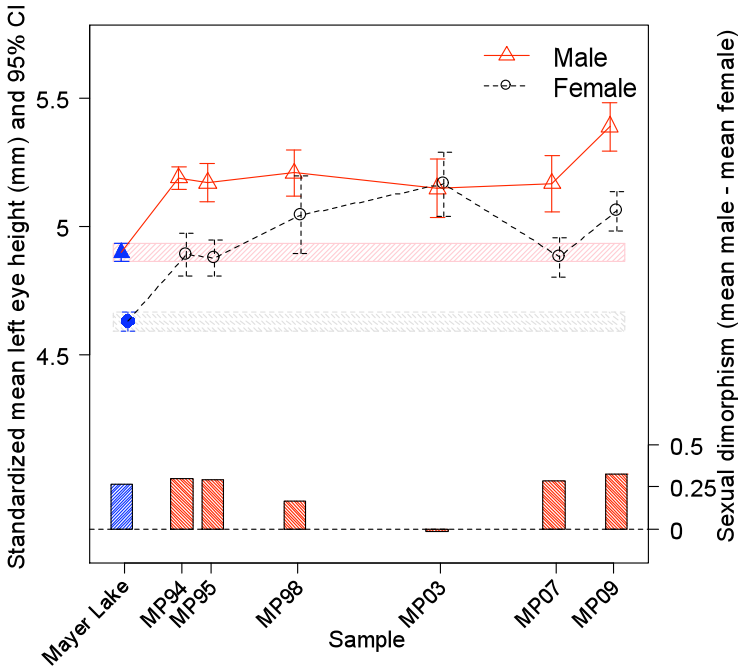


Figure 12: Standardized mean eye height in millimeters with 95% confidence intervals as well as bar graph illustrating extent of sexual dimorphism. All left eye height values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Left jaw length

There is little overall trend in differences of jaw length between Mayer Lake sample and Mayer Pond. Other than the most recent male sample in which Mayer Pond fish have significantly longer jaws (Tukey post-hoc comparison with ML males, p=0.04), none of the Mayer Pond samples are different from Mayer Lake (all other p>0.24). The degree of sexual dimorphism has not changed. Males have larger features than females (Figure 13).

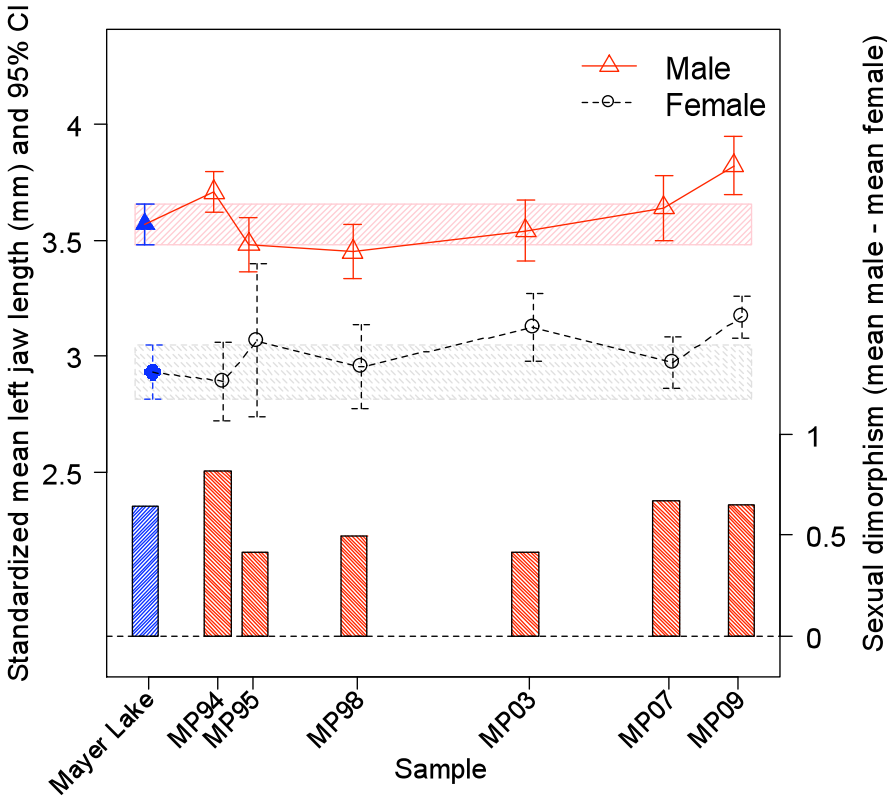


Figure 13: Standardized mean left jaw length (mm) with 95% confidence intervals as well as bar graph illustrating extent of sexual dimorphism. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Gill raker number

There are no significant trends in the number of gill rakers over time. Although the most recent MP09 male sample has reductions of one gill raker on the upper branch (Mayer Lake median count of 5, MP09 median count of 4), and the lower branch (Mayer Lake median count of 14, MP09 median count of 13) of the first left gill raker, there is no overall statistical difference over time (general linear model with Poisson error structure, $p>0.05$ for males and females, upper and lower branches; Figure 14).

With respect to sexual dimorphism, there appear to be differences between Mayer Lake and Mayer Pond. Four of the six Mayer Pond samples have females with a greater upper gill raker number count, while Mayer Lake and the other two Mayer Pond samples have the greater number of gill rakers in males. On the lower branch, the direction of dimorphism is the same with males having the greater gill raker count in all samples, but the magnitude of difference is almost fourfold higher in some of the Mayer Pond samples (mean difference of almost a whole gill raker in some Mayer Pond samples, but only 0.25 gill rakers in Mayer Lake). However, these extremes present in some of the early generations, disappear in the most recent Mayer Pond samples (Figure 14). There is no overall consistent trend on either the upper or lower branch.

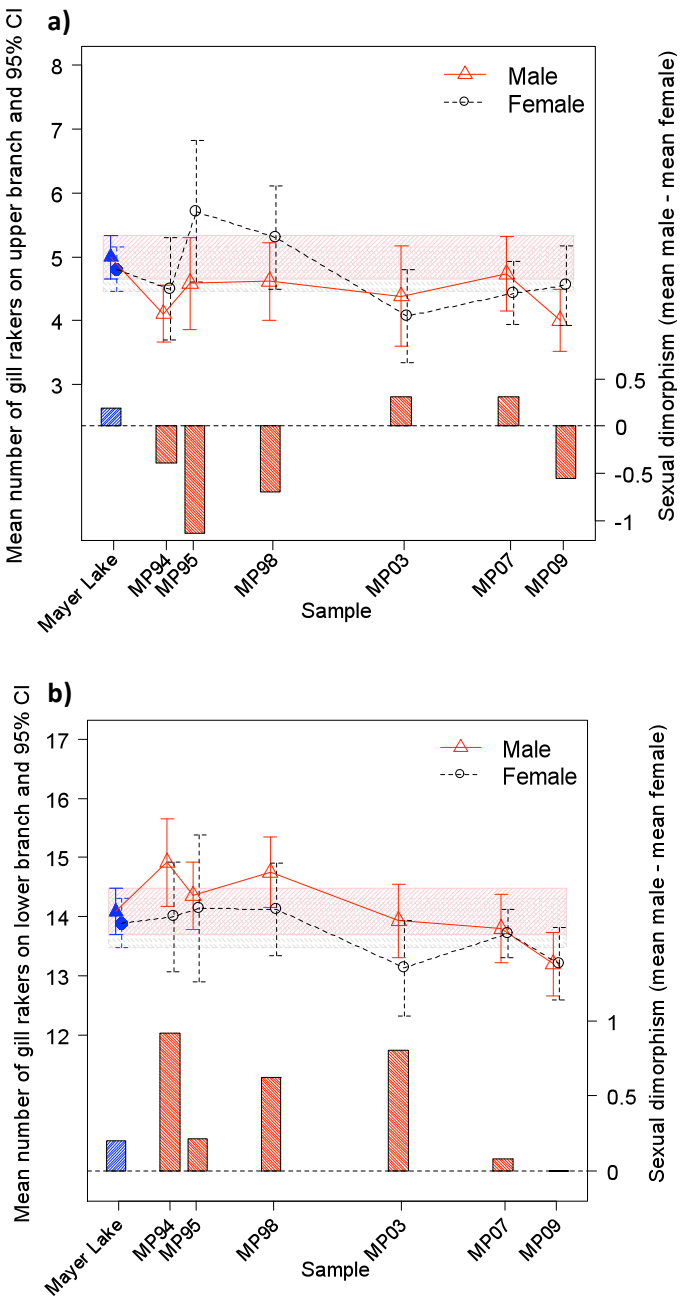


Figure 14: Mean gill raker number on the upper (a) and lower (b) branches of the first left gill raker with 95% confidence intervals as well as bar graph illustrating extent of sexual dimorphism. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Gill raker length

The lengths of the gill rakers have been significantly reduced in Mayer Pond as compared to Mayer Lake. This reduction is present in both sexes, and occurs immediately in the first generation and persists, (Tukey Post-hoc comparisons with ML in both sexes, all $p<0.002$; Figure 15). The other gill raker measurement, gill raker 3 bone length, is highly correlated (Pearson correlation =0.93, $p<0.001$), and shows similar trends.

The direction (males have longer gill rakers) of sexual dimorphism appears relatively constant across the samples (Figure 15).

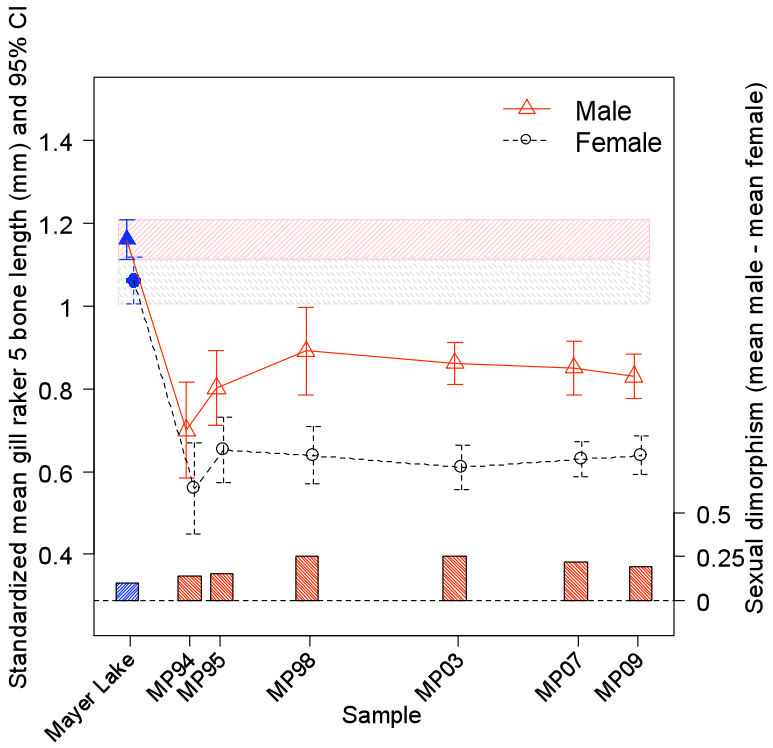


Figure 15: Standardized mean gill raker length (mm) for gill raker 5 bone length with 95% confidence intervals as well as bar graph illustrating extent of sexual dimorphism. All gill raker length values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Gill raker spacing

There appears to be a general trend that the gill rakers spacing is greater in Mayer Pond samples compared to Mayer Lake fish (Figure 16). However, only two of the male Mayer Pond male samples show significantly more widely spaced gill rakers (Tukey Post hoc comparison, $p=0.005$ for MP03, $p<0.001$ for MP09), and only the most recent female sample (MP09) shows a suggestive increase ($p=0.11$). Otherwise there are no significant differences (all $p>0.14$; Figure 16). Despite these relatively few statistical significant differences, the apparent increase in raker spacing in Mayer Pond is closely replicated in both sexes (Figure 16).

Additionally, there is more sexual dimorphism in the Mayer Pond samples compared to Mayer Lake fish. In Mayer Lake the sexes are indistinguishable for gill raker spacing, but in Mayer Pond males also have more widely spaced gill rakers than females, to varying degrees (Figure 16).

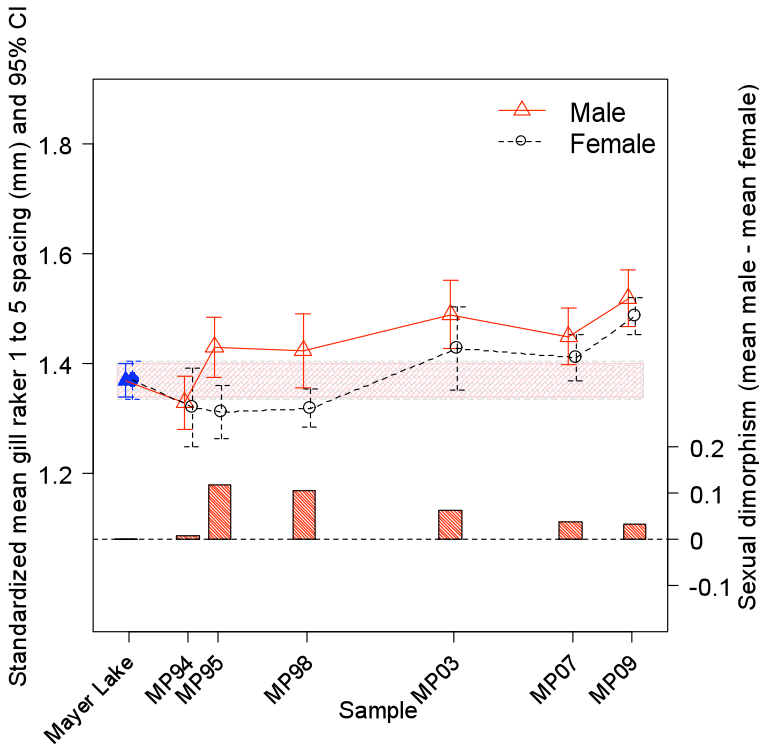


Figure 16: Standardized mean distance (mm) between the first and fifth gill rakers below the joint between the upper and lower branches of the first gill arch with 95% confidence intervals as well as bar graph illustrating extent of sexual dimorphism. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

3.3.4 Individual defensive structures

Lateral plate presence

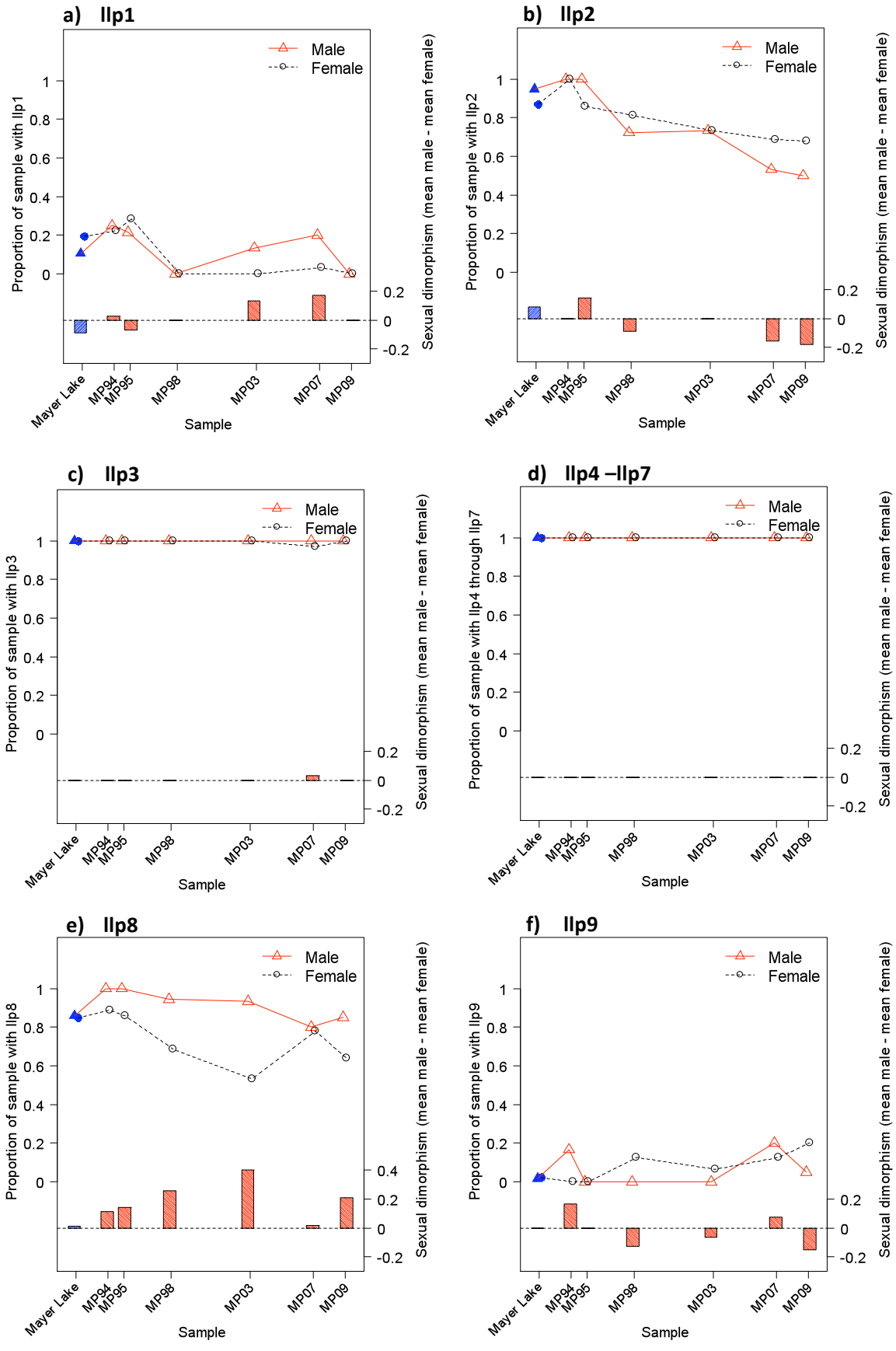
There is a decline in the number of anterior lateral plates over time in Mayer Pond. Although males do not exhibit significant trends in the first left lateral plate (general linear model with binomial errors, significance of the slope representing the proportion of fish with that plate over successive generations, p=0.21), females exhibit a significant decline in the proportion of a sample with that most anterior left plate (p=0.007). However, this plate is relatively uncommon, and present in only approximately 10-30% of the source population Mayer Lake (Figure 17a). The next lateral plate, position 2, occurs in approximately 90% of Mayer Lake fish, but by the most recent sample, MP09, it is only found in approximately 60% of individuals. This significant, progressive decline is seen in both males and females (all $p<0.02$; Figure 17b).

There is no change in the occurrence of the middle plate positions (positions 3-7). Only one female, from the MP07 sample, is missing left lateral plate 3. All other fish from all samples have these middle position plates (Figure 17c and Figure 17d).

There are, however, changes in the occurrence of the some of the posterior lateral plates, primarily in females. There is fluctuation in the 8th lateral plate, but no significant trends over time (all $p>0.11$ for males and females; Figure 17e). For the 9th lateral plate, although males show no change over time (all $p>0.23$), females show a slight, but significant increase in the presence of this plate the left side ($p=0.03$; Figure 17f). Similarly, males show no change in the presence of the 10th lateral plate (all $p>0.36$), but females once again show a small, but significant, increase in the proportion of fish that have this plate ($p=0.012$; Figure 17g). Out of the 272 fish examined, only four individuals had more than ten plates on the left side. Although these numbers are too small to analyze, it is interesting to note that all four fish were from the later Mayer Pond generations (one from MP03, one from MP07, and two from MP09).

Differences between the sexes are relatively subtle for most plate positions, but are quite pronounced in left lateral plate 8. The MP03 sample, in particular, shows an approximately 40% difference in the proportion of males and females that have llp8 (with males being more likely to have that plate). Although in the next sample, MP07, there is no difference between the

sexes, for llp8 it appears that males are more likely to have this plate position than females (Figure 17e).



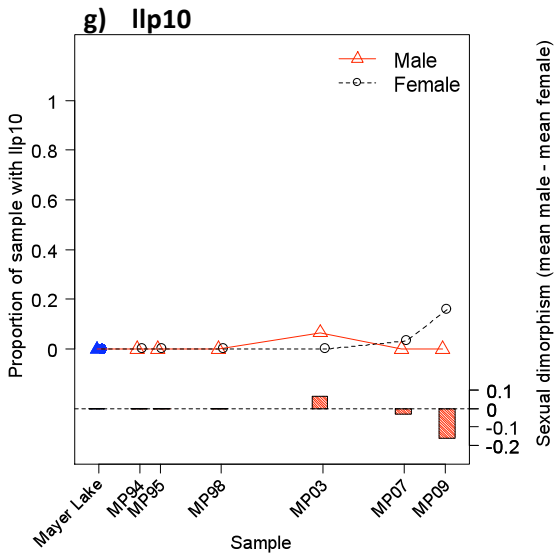


Figure 17: The proportion of a sample that has a specific left lateral plate (llp) in Mayer Lake compared to six successive samples from Mayer Pond (1994, 1995, 1998, 2003, 2007, and 2009) illustrating the potential extent and rate of changes in plate presence over 17 years since the 1992 transplant of stickleback from Mayer Lake to Mayer Pond. The following plate positions are shown: a) left lateral plate 1, b) left lateral plate 2, c) left lateral plate 3, d) left lateral plates 4 through 7,e) left lateral plate 8, f)left lateral plate 9, and g) left lateral plate 10. Plate positions follow those in Reimchen (1983). The degree of sexual dimorphism (male proportion – female proportion for a given sample) is represented by a bar graph at the bottom and corresponding axis at the right.

There are some differences if we look at the absolute asymmetry (absolute values of right-left plate count) in the total number of plates between Mayer Lake and Mayer Pond. In males, the number of symmetrical fish declines in Mayer Pond by the most recent 2007/2009 samples compared to Mayer Lake (75% of Mayer Lake males are symmetrical versus 51% of Mayer Pond males from the 2007/2009 samples; $X^2_1 = 4.6$, p=0.04). In females, there is no change in levels of plate symmetry (54% versus 51% of females are symmetrical for Mayer Lake and Mayer Pond, respectively; X^2 1 = 0.06, p=0.8).

If we examine the signed asymmetry (signed values of right-left plate count), we see further differences. While asymmetrical males tend to be right biased in both populations, there is an increasing proportion of left biased individuals in Mayer Pond males (Figure 18a). Females, although showing no difference in their degree of asymmetry between the two populations, do have greater variability in the total amount of asymmetry (range from -2 to 3) compared to males (which only range between -1 and 1; Figure 18).

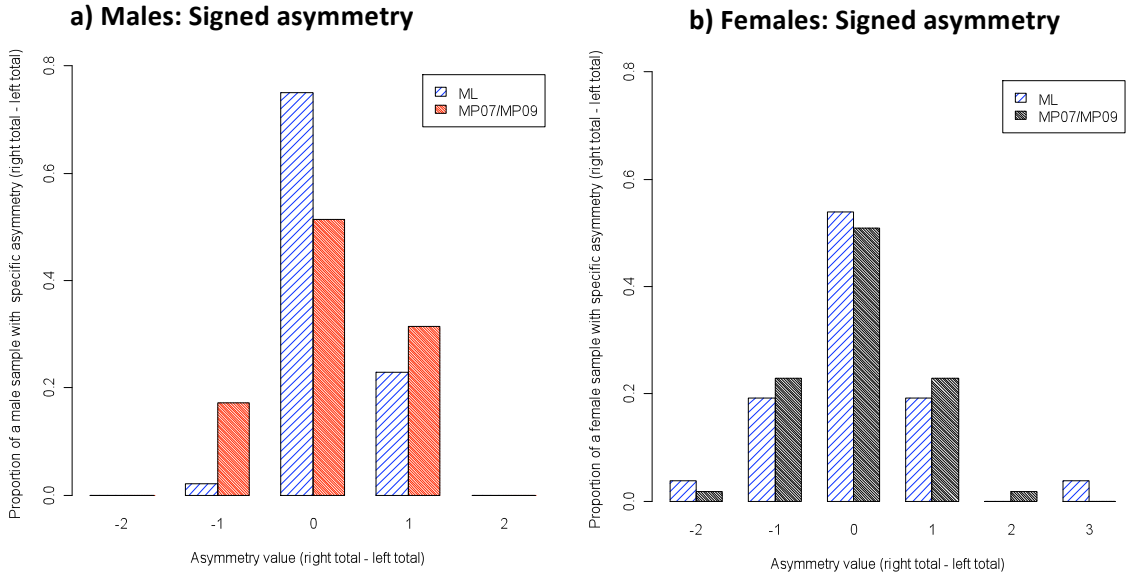


Figure 18: Signed asymmetry (total right side plate count minus total left side plate count) for a) males and b) females from Mayer Lake and the most recent 2007 and 2009 Mayer Pond samples. Mayer Lake is shown in blue, MP07/09 males are represented by more densely shaded red bars, and females are shown with more densely shaded black bars.

Asymmetry in plate presence, the difference between the right and left sides of individuals that have a plate on at least one side, was only examined for positions 2 and 8. Other plate positions were not examined as there were too few individuals with plates present, or because all individuals had all plates and there was no potential for asymmetry. For lateral plate 2 asymmetry, both populations are centered on a mean of zero, and no Mayer Pond samples are statistically different than the Mayer Lake samples for both males and females (Tukey post hoc comparison, all $p>0.6$). The same is true for lateral plate 8 asymmetry (Tukey post hoc comparison, all $p>0.39$). Males and females are also quite similar in their degree of asymmetry for these two plate positions, with the greatest difference being found in the MP07 sample for lateral plate 2, where males had a tendency towards greater lp2 presence on the right side, and females being more likely to have lp2 on the left side (Figure 19).

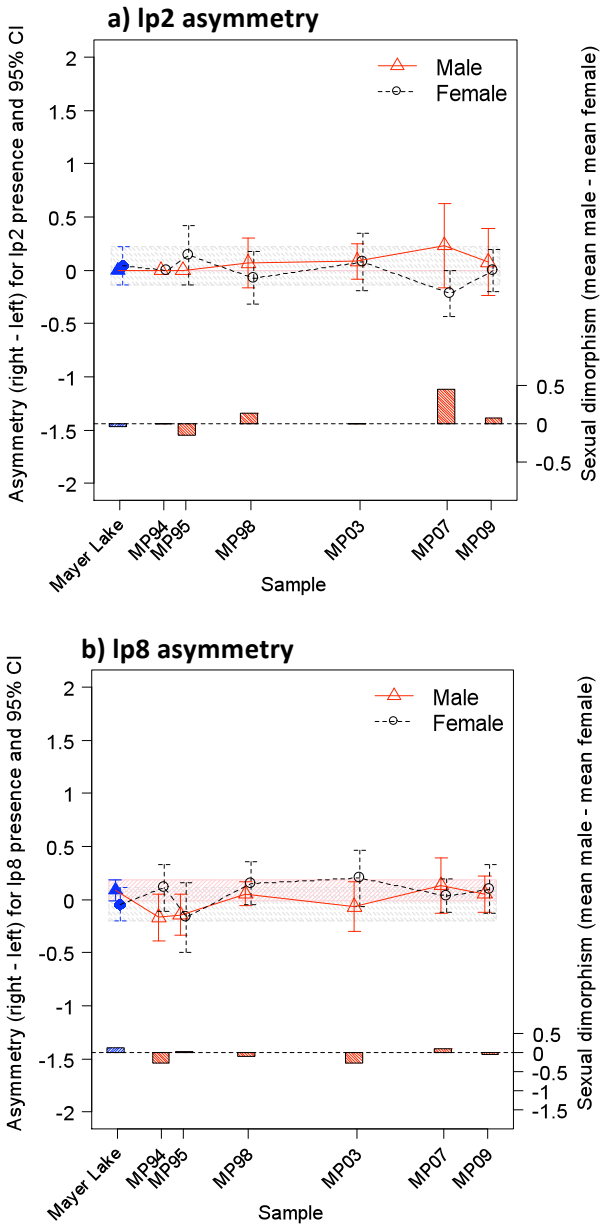


Figure 19: Mean lateral plate asymmetry for plate positions 2 (a) and 8 (b) with 95% confidence intervals, and the degree of sexual dimorphism for each sample shown with a bar graph along the bottom. Asymmetry is calculated as right- left for fish that have a plate on at least one side. Values of zero indicate a fish that is symmetrical, with plates on both sides, whereas positive values indicate a right side bias and negative values indicate a left side bias. (See Figure 10 for detailed figure layout and colour scheme explanation.)

It may seem that the loss of the 2nd lateral plate and the increase in some of the posterior plates represents a posterior shift in the positioning of the plates, but that is unlikely. Of the 36 fish that have a ninth lateral plate, only 7 (19%) are also missing the second lateral plate, and of all the fish that have a 10th lateral plate, or plates posterior to the 10th position, none are missing the second lateral plate. The addition of posterior lateral plates, primarily in females, would therefore represent an increase in the total number of plates, rather than a shift in the relative position of an equivalent number of plates. So overall, in addition to the loss of lateral plates, with an increase in the number of individuals having six instead of seven total plate counts, there is also an increase in the variation in the total plate count distribution, especially in females in Mayer Pond compared to Mayer Lake (Figure 20).

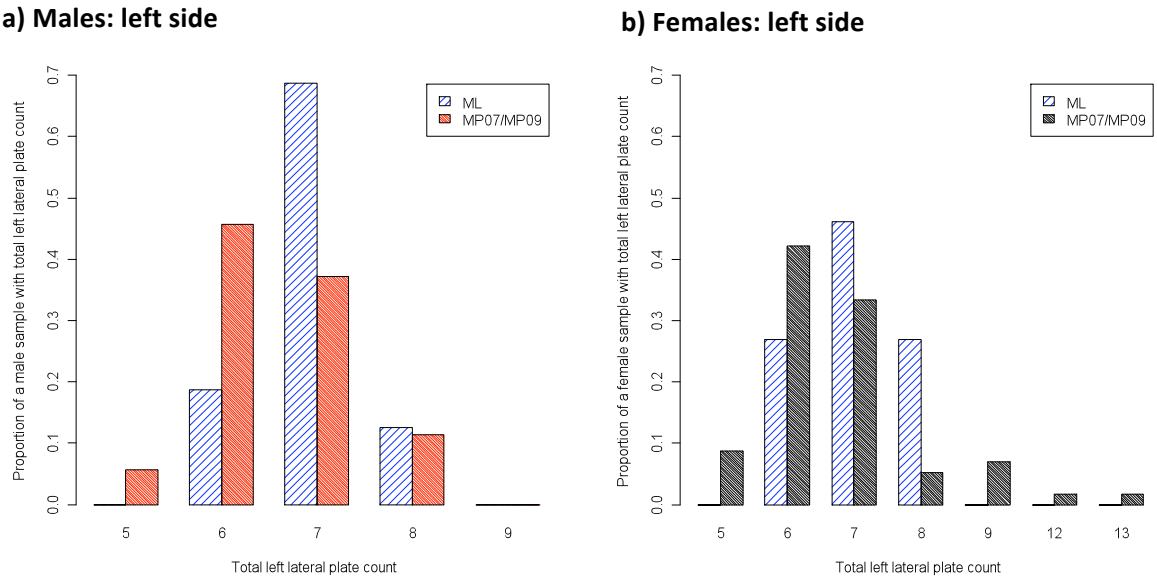


Figure 20: Histograms of Mayer Lake (ML) and Mayer Pond 2007/2009 samples (MP07/09) showing the total number of plates on the (a) left side of males, and (b) left side of females. The most recent Mayer Pond samples are pooled and compared to the source population, Mayer Lake, to see changes in distribution of total plate count after 15-17 years since individuals were transplanted from Mayer Lake to Mayer Pond. Mayer Lake is shown in blue, MP07/09 males are represented by more densely shaded red bars, and females are shown with more densely shaded black bars.

2nd Lateral plate dimensions

There is evidence of a reduction in the size of the left 2nd lateral plate, but primarily in males. Looking at the height of 2nd left lateral plate (see Figure 5 for example), all Mayer Pond male samples, with the exception of MP94 ($p=1$), are significantly smaller than Mayer Lake (Tukey post-hoc comparison, all $p<0.04$; Figure 21). The female samples do not show as clear a reduction trend in lateral plate 2 dimension. Two of the six samples, MP03 and MP07, show a significant reduction in left lateral plate 2 height (Tukey post hoc comparison with Mayer Lake $p<0.01$), but the other three samples are statistically equivalent to Mayer Lake (all $p>0.7$). The width of this plate is highly correlated with height (Pearson correlation = 0.6, $p<0.001$, and shows equivalent trends. Overall Mayer Pond males show the strongest evidence of a reduction in the size of the left lateral 2 plate.

Both the degree and direction of sexual dimorphism fluctuates in the Mayer Pond samples for the left lateral plate 2 height. The MP98 and the MP03 samples show the greatest degree of sexual dimorphism, but the directionality is opposite between the two (males have smaller plates in MP98, and larger plates in MP03).Overall, there is no clear divergence from Mayer Lake (Figure 21).

In both males and females, there are no significant differences in the degree of asymmetry in lateral plate 2 height between Mayer Lake and Mayer Pond individuals (Tukey post hoc comparison, all $p>0.27$). However, there does appear to be greater dimorphism in the extent of asymmetry in Mayer Pond fish with males, in general, perhaps becoming more right biased for left lateral plate 2 height in over time since transplantation (Figure 22).

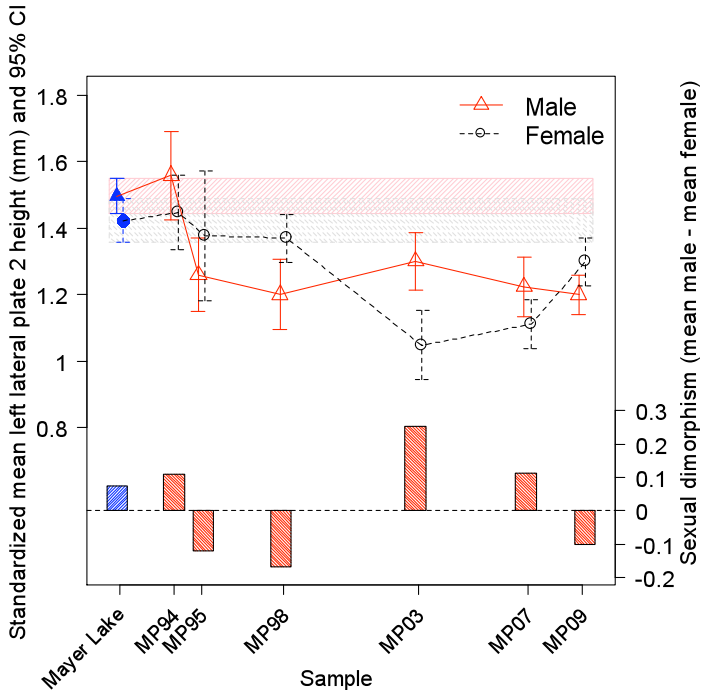


Figure 21: Standardized mean lateral plate 2 dimension (mm) with 95% confidence intervals and bars illustrated the extent of sexual dimorphism. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

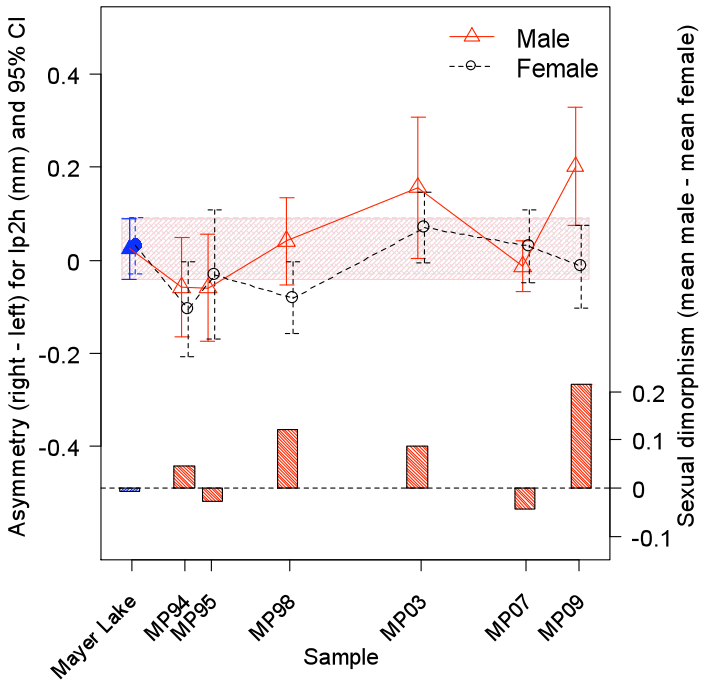


Figure 22: Mean asymmetry (right plate value – left plate value calculated for each fish) and 95% CI for a) lp2w and b) lp2h. (See Figure 10 for detailed figure layout and colour scheme explanation.)

3rd Lateral plate dimensions

As with the second lateral plate, there is evidence that the height of the 3rd lateral plate has also been reduced in Mayer Pond fish compared to Mayer Lake individuals. In males, four samples show a significant reduction in left lateral plate 3 height (post hoc comparison, all $p<0.001$). The other two samples, MP94 and MP03, show no statistical difference from the Mayer Lake baseline ($p=1$ and $p=0.6$, respectively; Figure 23). In females, three Mayer Pond samples, MP98, MP03, and MP07 show a significant reduction in left lateral plate 3 height (Tukey post hoc comparison, all $p<0.03$), while the other three samples show no statistical change ($p>0.4$; Figure 23). In the highly correlated left lateral plate 3 width (Pearson correlation = 0.43, $p<0.001$) no female samples can be statistically separated from Mayer Lake (Tukey post hoc test, all $p>0.08$). Overall, the evidence for a reduction in the 3rd lateral plate dimensions is strongest in male samples.

Mayer Pond seems to exhibit more sexual dimorphism than Mayer Lake, but the direction of difference between males and females fluctuates. Only two years, MP03, and possibly MP94 for lp3h, show differences sufficient that the error bars of the two sexes don't overlap, and in these cases males are larger than females (Figure 23).

With respect to left lateral plate 3 asymmetry, there are no statistically significant differences between Mayer Lake and Mayer Pond samples (Tukey Post hoc comparison, all ($p>0.3$ for both sexes). There are no clear trends in the sexual dimorphism of asymmetry for these two features (Figure 24).

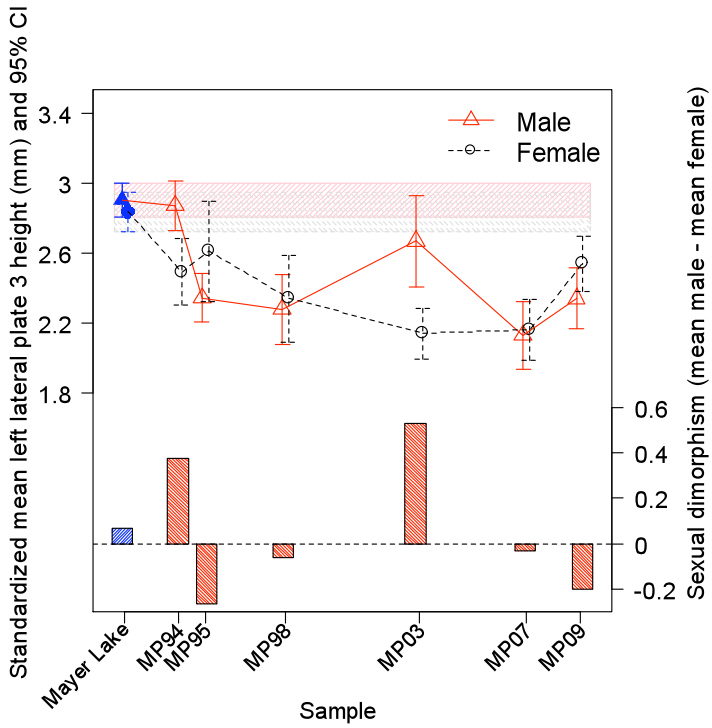


Figure 23: Standardized mean lateral plate 3 dimension (mm) with 95% confidence intervals and bars illustrated the extent of sexual dimorphism. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

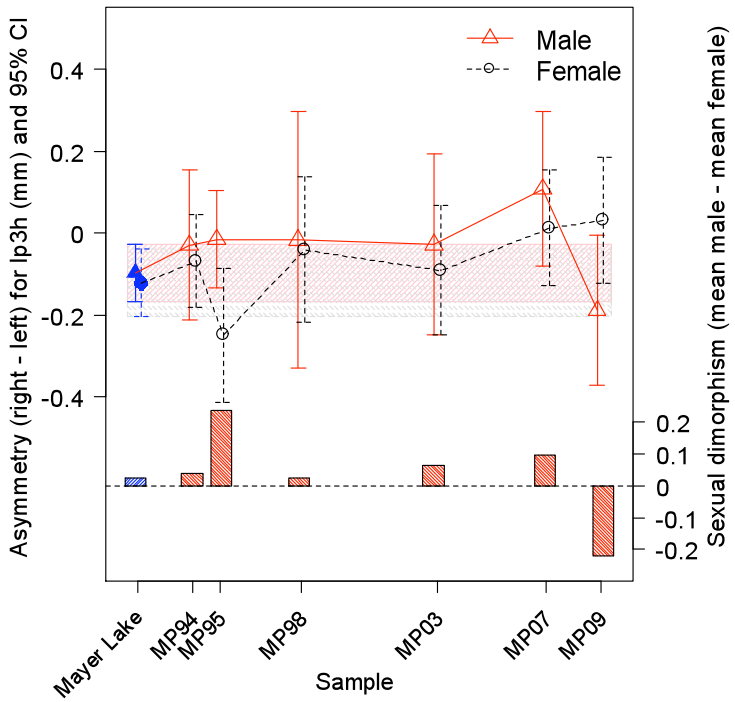


Figure 24: Mean asymmetry (right plate value – left plate value calculated for each fish) and 95% CI for a) lp3w and b) lp3h. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Spine length and width

There is a significant and immediate reduction of the length of the second dorsal spine in Mayer Pond versus Mayer Lake samples. All Mayer Pond fish, both male and female, have a significantly shorter second dorsal spines than their Mayer Lake counterparts (Tukey post-hoc comparison, all $p<0.01$; Figure 25). This trend is replicated in the very highly correlated (Pearson correlation =0.9, $p<0.001$) first dorsal spine length (Tukey post-hoc comparison of first dorsal spine length between Mayer Lake fish and all Mayer Pond samples, all $p<0.01$).

In general, there appears to be a greater sexual dimorphism in Mayer Pond samples. While Mayer Lake males and females are almost identical, with males being slightly larger, in almost all Mayer Pond samples females have longer dorsal spines, and the magnitude of this difference is greater (Figure 25).

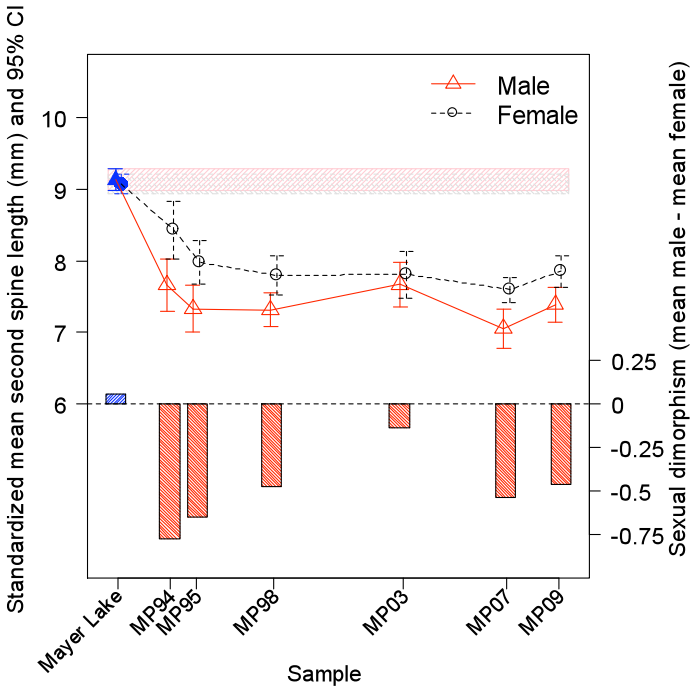


Figure 25: Standardized mean second dorsal spine length (mm) with 95% confidence intervals and a bar graph below illustrating the extent of sexual dimorphism (males – females) within each sample. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

In addition to a pronounced decrease in the length of dorsal spines in Mayer Pond, there is a corresponding decrease in the width of the first spine compared to Mayer Lake. All male Mayer Pond samples have significantly thinner first dorsal spines (Tukey post-hoc comparison, $p<0.001$), and, except for the MP94 female sample ($p=0.18$), all other female Mayer Pond samples show a similar reduction (all other $p<0.02$; Figure 26).

Unlike dorsal spine length, for dorsal spine width in all but one situation (MP09) males have larger features. In general, the degree of this dimorphism appears somewhat less in Mayer Pond samples compared to the Mayer Lake fish (Figure 26).

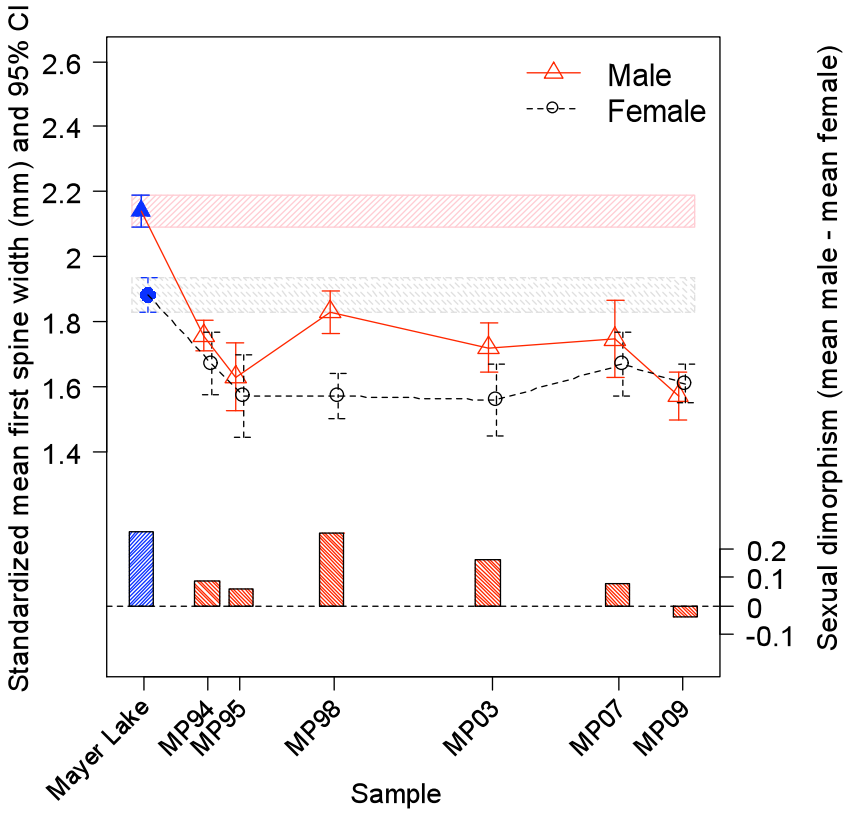


Figure 26: Standardized mean first dorsal spine width (mm) with 95% confidence intervals and a bar graph below illustrating the extent of sexual dimorphism (males – females) within each sample. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Like the dorsal spines, there is evidence of a reduction in the length of the left pelvic spine in Mayer Pond compared to Mayer Lake. All male Mayer Pond samples have significantly shorter pelvic spines (Tukey post hoc comparison, $p<0.02$). However, the change is not as apparent in females. MP07 shows a significant reduction ($p<0.001$) and MP03 is suggestively smaller ($p=0.08$), but the rest of the female samples show no change (all $p>0.58$; Figure 27).

Also similar to dorsal spines, females have longer pelvic spines than males in all of the Mayer Pond samples. Mayer Lake sexes are more equivalent in pelvic spine length (Figure 27).

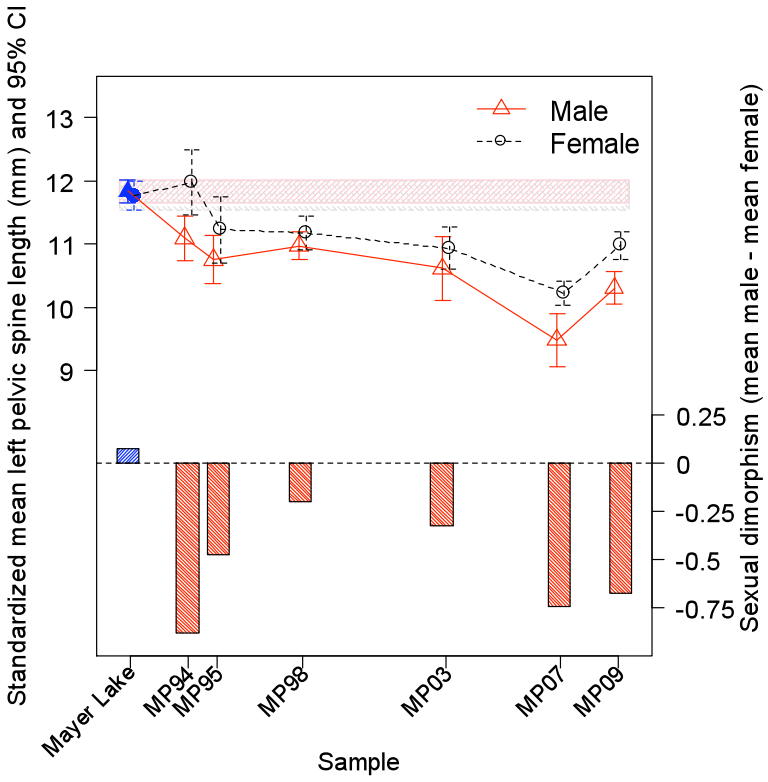


Figure 27: Standardized mean left pelvic spine length (mm) with 95% confidence intervals and a bar graph below illustrating the extent of sexual dimorphism (males – females) within each sample. All values have been standardized to those of a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Pelvic plate dimensions

There does not appear to be any consistent changes in the pelvic plate dimensions in Mayer Pond compared to Mayer Lake. Looking at the length of the pelvic plate, although all Mayer Pond males appear to have smaller pelvic plates than Mayer Lake males (Figure 28a), only one sample, MP07, shows a significant reduction ($p<0.001$) and another, MP98 shows a suggestive reduction ($p=0.06$). The other male samples show no change (Tukey post-hoc comparison, all other $p>0.16$; Figure 28a). In females the MP07 sample also shows a reduction in pelvic plate length ($p=0.04$), but the rest of the female samples show no difference between Mayer Lake and Mayer Pond (Tukey post-hoc comparison, all $p>0.12$; Figure 28a). Furthermore, if we look at the overall area of the pelvic plate, although there is a reduction in the MP95 male sample ($p=0.008$) there is no change between any of the other Mayer Pond samples and Mayer Lake (all $p>0.10$ for males and females; Figure 28b). (Note: this measurement was not carried out for the intermediary samples due to lack of trends and insufficient confidence in the repeatability of the measurement.) Although it is interesting that MP07 showed such a reduction in length, but not overall area (indicating that this sample may have had shorter, fatter, pelvic plates), with regard to the overall comparison of Mayer Lake and Mayer Pond there are no strong indications of changes in the dimensions of the pelvic plate.

For both features, but particularly pelvic plate length, females exhibit greater values than males in the Mayer Pond samples. This direction is also found in Mayer Lake fish, although the magnitude of difference may be slightly less (Figure 28). The oscillations in pelvic plate length across the MP03 to MP09 samples are replicated by both sexes (Figure 28a), further supporting the accuracy of these measurements.

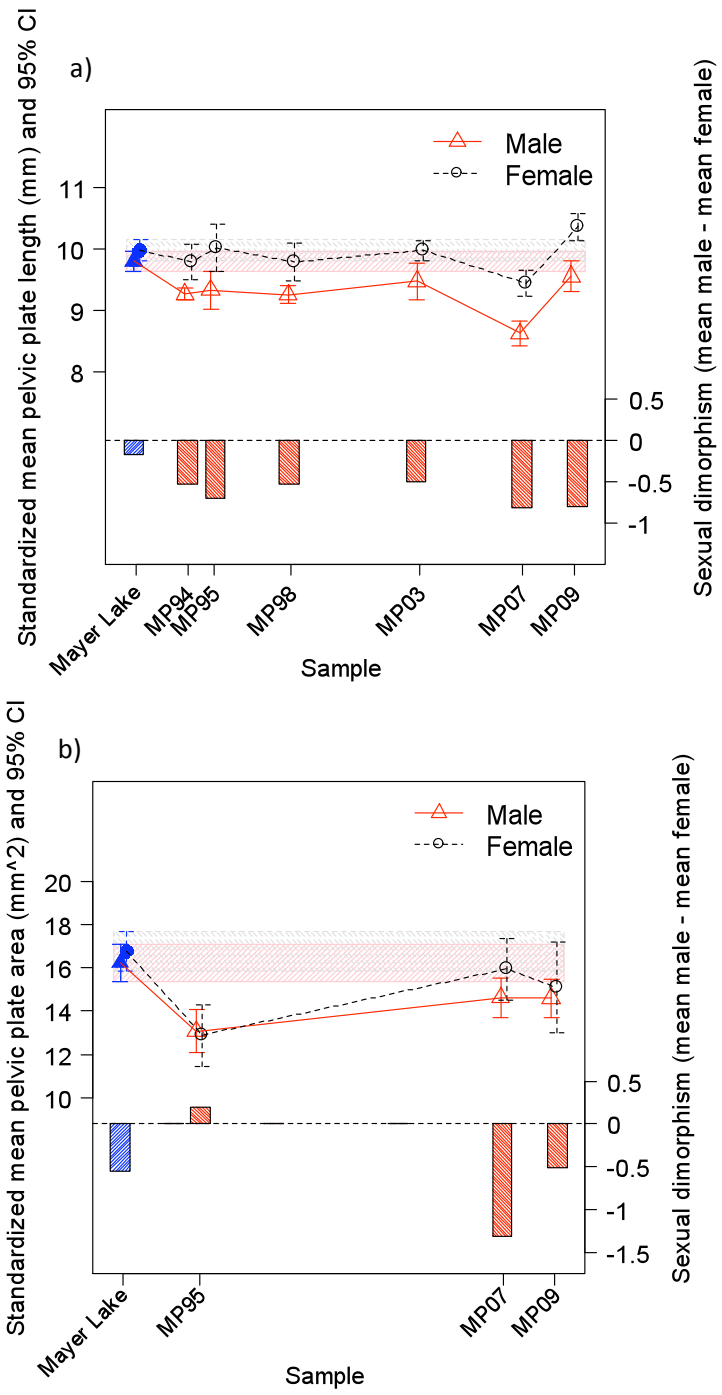


Figure 28: Standardized mean (a) length (mm) and (b) area (mm^2) of the pelvic plate with 95% confidence intervals, as well as bar graphs showing the extent of sexual dimorphism for these two features. All values have been standardized to those found in a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Ascending process dimensions

There is a significant reduction in the area of the left ascending process of Mayer Pond samples compared to Mayer Lake. With the exception of MP94 (males and females, $p>0.09$), and the MP09 females whose left ascending process areas that are not quite significantly smaller ($p=0.056$), all other Mayer Pond samples, male and female, show a significant reduction in ascending process compared to Mayer Lake (Tukey post-hoc comparison, all $p<0.05$; Figure 29a).

The degree of sexual dimorphism, while many times larger in Mayer Pond samples compared to Mayer Lake, does not represent considerable differences between males and females if the degree of overlap in the error bars for each sex within each sample is also considered. However, in general males tend to have larger ascending processes than females (Figure 29a).

With respect to asymmetry in ascending process area, there are no significant differences between Mayer Lake and Mayer Pond samples (Tukey post hoc comparison, all $p>0.6$). However, there may be differences in the sexual dimorphism of asymmetry in Mayer Pond, with Mayer Pond females typically being more left biased (larger ascending processes on the left side), and Mayer Pond males being more right biased (Figure 29b).

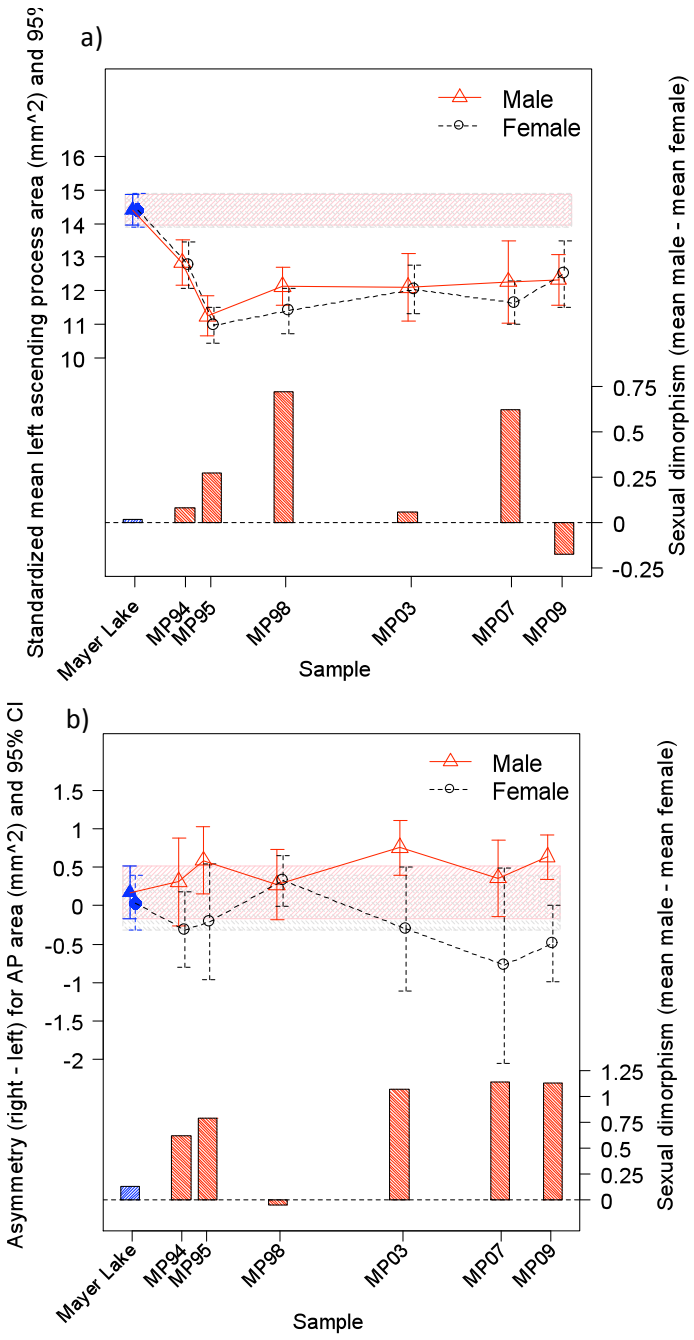


Figure 29: Standardized mean (a) left ascending process area and (b) asymmetry in ascending process area and 95% CI, with bar graphs below illustrating the extent of sexual dimorphism in both measures. All ascending process area values have been standardized to those found in a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

Looking at the measurements of the height of the ascending process, we can infer whether this reduction in the overall ascending process area seen in Mayer Pond compared to Mayer Lake was the result of a decrease in height or width. In males, there is no change in the height of the compared to Mayer Lake (Tukey post-hoc test, all $p>0.09$; Figure 30a). In females, two samples, the MP03 and MP09 sample, are equivalent to Mayer Lake (for both samples and both left and right sides, $p>0.13$). In the other samples, Mayer Pond has significantly shorter ascending processes than Mayer Lake (Tukey Post hoc comparison, $p<0.05$ for all other samples). It would seem that in males, the reduction in ascending process is the result of a decrease in width, while in females a reduction in both width and height may contribute to the overall smaller ascending process area of Mayer Pond samples compared to Mayer Lake samples.

The degree of sexual dimorphism is greater in Mayer Pond samples compared to Mayer Lake fish. Mayer Lake males and females are roughly equivalent, but in Mayer Pond males are consistently larger than females. However, the magnitude of this difference is not consistent over time and declines by the most the most recent MP09 sample (Figure 30a).

With respect to asymmetry, there is one suggestive change, but overall no statistical difference between samples from the two populations. The male MP98 sample is suggestively less right biased than the Mayer Lake baseline (Tukey post hoc comparison, $p=0.06$). Otherwise there is no statistical difference between the asymmetry of Mayer Pond and Mayer Lake samples for males and females ($p>0.35$), although it is interesting to note that the Mayer Lake sample is not centered on zero asymmetry, but instead shows a distinctive right bias in the height of the ascending process (Figure 30b). In the Mayer Pond samples, females are more likely to reflect this right bias compared to males, as can be seen in the direction of sexual dimorphism for this morphological measure (Figure 30b).

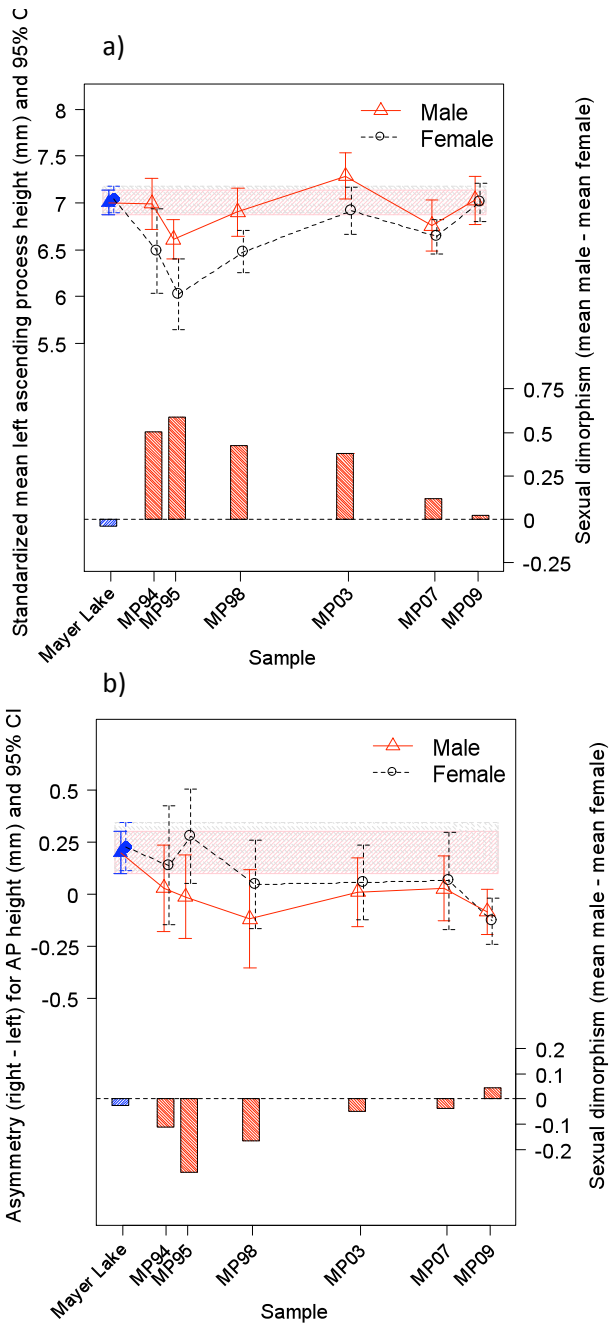


Figure 30: Standardized mean (a) left ascending process height and (b) asymmetry in ascending process height with 95% CI, as well as bar graphs below illustrating the extent of sexual dimorphism in both measures. All ascending process height values have been standardized to those found in a 60 mm fish. (See Figure 10 for detailed figure layout and colour scheme explanation.)

3.4 Discussion

I expected a number of morphological changes in Mayer Pond fish from the Mayer Lake ancestral population. As Mayer Pond represents a more benthic-type habitat, with plenty of vegetation, little open water, and a benthic prey field, I expected Mayer Pond fish to transition to a benthic-type form, specifically having a deeper body, larger eyes, shorter jaws, and fewer and shorter gill rakers, than the Mayer Lake samples. Also, since the Mayer Pond habitat has invertebrate, rather than vertebrate, predators compared to Mayer Lake, I predicted that defensive structures including any bony armour structures (lateral plates, ascending process, pelvic plate), dorsal spines, and pelvic spines would be reduced in size (and possibly presence for lateral plates).

Using a multivariate principal component analysis which identifies the features that best explain the variation in the data, I found that a feeding structure – gill raker length – and a defensive feature – dorsal spine length – explained the most variability in the data, and best differentiated the Mayer Lake and Mayer Pond samples. Furthermore, this statistic showed that both features were reduced in Mayer Pond fish as I had expected. I also examined each feature individually using Tukey post hoc comparisons, or general linear models with the appropriate error structure, to compare the divergence of each Mayer Pond sample from the Mayer Lake baseline.

3.4.1 Feeding structures

Two feeding features, eye size and gill raker length, are different in the transplant population Mayer Pond compared to the source population Mayer Lake, while one feature, maximum depth, shows different trends over time, and the other features, jaw length and gill raker number, remain unchanged. Specifically, fish from Mayer Pond have larger eyes and shorter gill rakers than those from Mayer Lake. Although there is some evidence that the change in eye dimensions may represent rapid selection for a heritable trait, the immediate significant and persistent reduction in gill raker length of Mayer Pond fish most likely represents environmental influence and phenotypic plasticity.

Differences in stickleback feeding features have been most thoroughly examined in relation to the unique species pairs of stickleback that occur in some lakes (McPhail 1992, Schluter and McPhail 1992, Cresko and Baker 1996, Hatfield 1997, Peichel *et al.* 2001). Typically,

'benthic' individuals are characterized by feeding primarily on benthic prey and have a morphology consisting of fewer and shorter gill rakers, a larger mouth, and deeper bodies than sympatric plankton-eating 'limnetic' individuals (Schluter and McPhail 1992). A larger eye diameter has been considered 'benthic' by some (Reimchen and Nosil 2006), and 'limnetic' by others (Cresko and Baker 1996).

Of these features, gill raker number and length are often the best discriminators of these two morphotypes (Schluter and McPhail 1992, Bolnick 2004, Berner *et al.* 2008, Bolnick and Paull 2009). Furthermore, both of these features are considered highly heritable, with heritability estimates (the proportion of variation in the offspring explained by genetic, rather than environmental, variability) ranging from 0.36 (Schluter 1996a) to 0.51 (Aguirre *et al.* 2004) and 0.58 (Hagen 1973). My observations of no change in gill raker number, but an immediate and apparently plastic decrease in gill raker length, may provide a relevant addition to studies where high heritability of gill raker features is assumed and used for interpreting the strength and type of selection (Bolnick 2004), as well as the importance of natural selection for biological diversification (Berner *et al.* 2008).

'Benthic' type fish are characterized by deeper bodies (Schluter and McPhail 1992), and since Mayer Lake is a more limnetic environment (considering both size and prey availability) than Mayer Pond I expected fish would transition to a deeper body form following transplant. Initially however, the maximum depth of individuals was reduced in the earliest samples from Mayer Pond. Only in the most recent sample from 2009 do Mayer Pond fish have significantly deeper bodies. A previous student examining these two populations postulated that this reduction may be a phenotypically plastic response to limited food resource and poor nutrition (Parker 2004). Her samples ended in 2002 and she did not therefore see the increase in body depth from 2003 onwards until by 2009 Mayer Pond fish were significantly deeper than Mayer Lake individuals. Although it is possible that processes of succession increased the quantity or quality of food resources in the most recent sampling years from Mayer Pond, as the stickleback population has grown considerably beyond the founding 100 individuals, I do not believe increased access to food resources is the best explanation for why I saw an increase in body depth in the most recent samples. Perhaps her other explanation, that the transplant contained unusually thin individuals from Mayer Lake (Parker 2004), is accurate, and my results demonstrate a selective pressure for a deeper bodies in Mayer Pond that only recently resulted

in sufficient change for Mayer Pond individuals to be significantly deeper than the conventional Mayer Lake residents.

The absence of considerable changes in gill raker number may indicate methodological issues, or it may reflect this features high heritability. Extracting the full gill arch was methodologically challenging and some of the samples had to be excluded because it was not clear that I had been successful at removing the entire gill arch. As the differences between Mayer Pond and Mayer Lake are subtle, and only appear in the most recent sample (interestingly one of the largest samples), this reduced dataset, or the increased variability from inaccurate counts, could have prevented the detection of any more changes. However, it is also possible, and perhaps more likely, that over this short time frame of 17 years there has been insufficient time for the selective pressures in this environment to considerably influence what has been demonstrated as a highly heritable trait (Hagen 1973, Aguirre *et al.* 2004).

The significant and immediate reduction in gill raker length seen in the Mayer Pond transplant is particularly interesting, especially in relation to the broader literature. Of the before mentioned reports of gill raker heritability, only one (Schluter 1996a) explicitly includes an estimate for gill raker length, the others are only for gill raker number (although most are often cited to back up specific assumptions of gill raker length heritability). Overall, the heritability of gill raker length is less well documented than for gill raker number, and, from this present study, it would appear that length may be significantly influenced by environmental conditions, something which has previously received little consideration. Immediately in the first samples from Mayer Pond the gill rakers are significantly shorter, and this reduction persists with little variability in the remaining 15 years of samples. Either the founding population from Mayer Lake had uncharacteristically short gill rakers for the population, or selection was so intense that only those with short gill rakers survived in the first few years, or, most parsimoniously, some environmental feature induced by some mechanism (e.g. developmental changes caused by different diet - see Day *et al.* 1994, Day and McPhail 1996) a phenotypically plastic reduction of this feature. Furthermore, the prey of these two populations mirrors what commonly differentiates 'benthic' and 'limnetic' forms (Schluter and McPhail 1992), with the Mayer Lake prey field being dominated by Cladocerans and Mayer Pond diet containing Ostracods (see Chapter 2). This matching of 'benthic'-type morphology and prey in Mayer Pond, but the apparent plasticity of the response of gill rakers, may be important for understanding how these changes occur in other situations.

Feeding features have also a demonstrated sexual dimorphism, likely associated with different ecological niches between the sexes (Reimchen and Nosil 2004). In all feeding structures examined males had larger features than females, concordant with results from previous work (Reimchen and Nosil 2006). Interestingly, in some cases, particularly gill raker length and spacing, the dimorphism appears greater in Mayer Pond compared to Mayer Lake. This may reflect a greater range of ecological opportunities, and potential niche partitioning between the sexes, in this new habitat(Reimchen and Nosil 2004).

3.4.2 Defensive structures

The lateral plates of stickleback have been one of the most examined defense structures in this species (Hagen and Gilbertson 1973b, Moodie and Reimchen 1976b, Gross 1978, Reimchen 1983). While earlier investigations focused on gross differences in plate morphs (low, partial, completely plated (Hagen and Gilbertson 1973a), subtle differences in the specific number of plates within a morph (particularly low plated) have a demonstrated ecological role (Reimchen 1992, Reimchen 1994) and have received increasing attention (McPhail 1992, Kristjansson 2005, Knecht *et al.* 2007, Reimchen *et al.* 2008). In the last ten years with the development of a genome wide linkage map for stickleback (Peichel *et al.* 2001), the specific genetic mechanisms controlling the fine tuning of plate number have also been identified (Colosimo *et al.* 2004, Knecht *et al.* 2007). The progressive loss of the second lateral plate in the Mayer Pond population after transplantation from Mayer Lake fits both the ecological and the genetic context previously described (Bell 2001).Specifically, as Mayer Pond lacks predatory fish, but has invertebrate predators, the progressive loss of the anterior lateral plates over successive generations may reflect the selective process that has led to the endpoint observed in other fish predator free localities (Bell and Foster 1994).

Unlike the changes in gill raker length, which appear to be plastic, the progressive loss of anterior lateral plates is more appropriately interpreted as heritable changes over successive generations. Although possible that this feature is environmentally influenced, and progressive changes over time reflect changing environmental conditions (e.g. such as those found as the pond undergoes succession), this feature has been identified as having high heritability (Hagen 1973, McPhail 1992, Hatfield 1997, Bell 2001) and the genetic mechanisms of plate number control have been identified (Colosimo *et al.* 2004, Colosimo *et al.* 2005, Knecht *et al.* 2007). Specifically, there is a major gene on linkage group 4 (LG4) that determines plate number, with

individuals homozygous for the 'benthic' allele being low plated (Colosimo *et al.* 2004) like the populations in Mayer Lake and Mayer Pond. There are also up to five modifier alleles which fine tune the specific number of lateral plates, and can influence plate number by up to 15 plates per side (Colosimo *et al.* 2004, Knecht *et al.* 2007). Given this other work on the high heritable components and genetic control of this trait, I interpret the progressive loss of anterior lateral plates as being driven by natural selection and caused by genetic changes in the Mayer Pond population.

In addition to the loss of anterior lateral plates, there is also an increase in the presence of posterior plate positions, particularly in females, in the most recent generations of Mayer Pond. However, this is not a shifting of plates from the anterior to the posterior of the fish. Individuals with additional posterior plates have also retained the anterior plates. There is therefore an increase in the variance of total plate count over time in Mayer Pond. This may represent a sort of character release. Free from selection by predators for specific plate counts, this population may be able to express increased variance in this defensive structure without associated fitness costs (Bell 2001, Reimchen and Nosil 2006). Individuals that previously wouldn't have survived with this suboptimal phenotype in Mayer Lake can survive in Mayer Pond. This increased range of variability in plate count may therefore be a more accurate representation of the original frequencies in young stickleback from both Mayer Lake and Mayer Pond than what would be inferred by sampling the adults from Mayer Lake.

A more subtle reflection of loss of plates may be found when examining the dimensions of the remaining third lateral plate. The remaining anterior lateral plate 3 is slightly, but significantly, smaller in some Mayer Pond samples compared to Mayer Lake. Although it is not lost, perhaps this reduction in size represents an additional reduction of energetic investment in an unnecessary structure. Loss of lateral plates also tends to proceed with plate 3 being lost after plates one and two (Reimchen 1983). Perhaps in sufficient time this plate will also be fully lost, rather than simply reduced, in Mayer Pond stickleback. Although other work (Colosimo *et al.* 2004) found that alleles responsible for a decrease in total plate number also reduced overall plate size, the variability across samples and between sexes make interpreting the heritable components of plate dimensions from my data difficult. However, as one of the samples that shows a significant reduction is from 1995, and probably represents the first generation in this new environment, it would seem there is flexibility in the expression and development of this feature than can be environmentally influenced.

Dorsal and pelvic spines are other important defensive structures that show a change in Mayer Pond samples compared to Mayer Lake fish. Erect spines increase the maximum diameter of the fish, an effective first deterrent against gape limited piscivores, and will also puncture predator's mouths should they be able to capture the fish (Hoogland *et al.* 1956). They are also quite strong and can resist tremendous strain when supported by the lateral plates. Spines significantly improve both the likelihood of escape and also survival of captured stickleback (Reimchen 1983) . However, they are not always advantageous. Invertebrate predators may use the spines to restrain captured stickleback (Reimchen 1980), and (Marchinko 2009) found that selective pressures from invertebrate predators resulted in significant selection on the genes controlling spine length and an overall reduction in spine length over only a few generations. In general then, fish predator dominated ponds have stickleback with longer spines, but in small lakes where fish predators are absent, and invertebrate predators dominate, fish have shorter, or maybe even no dorsal spines (Reimchen 1980, Nosil and Reimchen 2005). There is even spatial and temporal correlation between spines and proximity to invertebrate predation, with fish inhabiting habitat where there is an increased likelihood of invertebrate predation (e.g. near shore in benthic habitats or during summer when larvae are more active) having reduced spines than individuals who are less likely to encounter this form of predation (Reimchen and Nosil 2002). Mayer Pond is a small pond, with no fish predators, but many invertebrate predators, so it is not surprising that we see a significant reduction in spine length and width of Mayer Pond individuals compared to the Mayer Lake source population.

However, the full and significant reduction of both dorsal and pelvic spine length, and dorsal spine width, is seen immediately in the first generations of Mayer Pond colonizers, and persists unchanged over subsequent years since transplantation. Like the immediate change in gill raker length, there are three possible explanations: 1) the original transplant contained an unusually high proportion of short-spine individuals compared to the distribution in Mayer Lake; 2) the selective pressures in Mayer Pond were so strong that only the short-spine individuals from the transplant survived and bred, and/or the only fish that survived that first generation to be sampled in 1994 were those with short spines; 3) spine length is phenotypically plastic, and reduced by some environmental, rather than genetic feature (although note that the potential to respond to environmental cues could have a genetic basis). Although not well studied, the genetic control of spine length has been described (Hatfield 1997, Peichel *et al.* 2001, Colosimo *et al.* 2004), and high heritability of this trait has been assumed when investigating differences

in selection between populations (McPhail 1992). Furthermore, the first dorsal spine maps to completely different genetic regions than does the second dorsal spine and the pelvic spine (Peichel *et al.* 2001), so the equivalent and immediate change in the first and second dorsal spines, as well as the pelvic spine, may also support a plastic, rather than a genetic based, reduction in the case of the Mayer Pond population. Although I do not know the mechanism (lack of predatory olfactory cues, physical abrasion with the increased vegetation in Mayer Pond) of this environmentally induced response, it would seem clear that there is much more plasticity in this feature than previously thought. This not only has important implications for interpreting selection when comparing this feature in other populations, it indicates a tremendous potential for morphological flexibility in this species. However, to more conclusively distinguish between mechanisms 1-3, we would need to examine lab-raised fish (see Chapter 4).

There also appears to be a phenotypically plastic reduction in the size of the ascending process, as indicated by the immediate and persistent reduction of this feature in all of the Mayer Pond samples. This feature provides important support when buttressing the pelvic spine against compression by predators. It is reasonable that this support is not as important in a predatory fish free environment such as Mayer Pond, and the reduction of this energetically costly feature is advantageous. The ascending process has not received much attention in previous morphological studies, and its genetic control has not been investigated. However, similar to plate dimension and spine length, this ossified defense structure is presumably less necessary in Mayer Pond, and is reduced, but it is reduced immediately upon introduction to this novel environment.

In addition to predation, a variety of mechanisms have been postulated as to the repeated loss of ossified structures such as lateral plates as marine stickleback colonized in freshwater lakes (Peichel 2005). Giles (1983b) found that lower calcium levels resulted in fish with reduced plates and spines and Marchinko and Schluter (2007) found that fish with fewer lateral plates grew faster with lower salinity levels (presumably also because of differences in calcium availability). Although calcium levels are higher in Mayer Pond compared to Mayer Lake (as indicated by conductivity; T.E. Reimchen, personal communication), perhaps another abiotic feature in Mayer Pond is different compared to Mayer Lake resulting in a reduction of ossified structures such as the lateral plate dimensions, dorsal and pelvic spine length, and ascending process area. Or, perhaps these conditions are equivalent in Mayer Lake, but individuals with reduced features do not survive to adulthood and are therefore not sampled. A third possibility

is that some predatory cue, such as olfactory cues from predatory cutthroat trout, environmentally induce larger defensive structures – similar to the classic example of inducibly thicker calcified shells in *Nucella lamellosa* in response to predatory olfactory cues (Palmer 1992, Bourdeau 2010). (However, see difference in spine length of lab raised fish under identical conditions in Chapter 4.)

The direction of sexual dimorphism in defensive structures depends on which feature is examined. In the ascending process area and spine width, similar to all the feeding features, males are larger than females. However, for all the spine lengths (both dorsal and pelvic), females are larger than males. Other than spine length, the sexual dimorphism of these structures has not been investigated. One study examining widespread geographic correlations found a general trend for males to have larger spines (Reimchen and Nosil 2006), but in some specific localities males had shorter spines than females (Reimchen 1980, Reimchen and Nosil 2004) similar to what I have found. Another study by Kitano (2007) examining sexual dimorphism across a number of different populations found the direction and extent of dimorphism to vary between populations, with some having females with larger spines than males.

One aspect of asymmetry examined in sticklebacks is in the number of lateral plates on the left and right sides of the fish (Moodie and Reimchen 1976b, Bergstrom and Reimchen 2000). Differences in niche have been associated with different asymmetries (Reimchen *et al.* 2008), and laterality in predator hunting behaviour may also possibly drive ecological separation of asymmetry types (T.E. Reimchen, personal communication). In both Mayer Lake and Mayer Pond females have greater variability in the degree of asymmetry attained, perhaps indicative of greater niche width, but there is no difference between the two populations. In males, however, Mayer Pond is much more likely to have asymmetrically plated individuals. If these asymmetries represent different niches, perhaps Mayer Pond offers greater ecological opportunities. If, however, asymmetry reflects developmental instability (Palmer and Strobeck 1986), then the reduced vertebrate predation in Mayer Pond may mean that these less fit individuals are more likely to survive and be represented in the sampled adult population.

Over 16 years since the original transplant there are changes in the feeding and defensive structures in Mayer Pond. The gill rakers are shorter and defensive structures such as lateral plate count and size, dorsal and pelvic spine length, and ascending process area, are reduced. As Mayer Pond is an environment where there are no predatory fish, but there are

predatory insects, the reduction of these defensive features makes ecological sense – either as a response to lose unnecessary, and costly features, or as protection against invertebrates which may use spines to capture stickleback. One of these traits, the loss of anterior lateral plates, appears to represent changes in gene frequency over time, but the rest occur immediately in the first generations and persist, perhaps indicative of an environmentally induced, phenotypically plastic change. Alternatively, it is possible that a genetic bottleneck or very intense selection in the first generation of fish in Mayer Pond has caused these immediate changes. Examining fish reared in equivalent conditions is necessary to discriminate between these possible mechanisms.

Chapter 4: Evaluating the morphological divergence of Mayer Pond stickleback from the Mayer Lake population using lab-raised fish

4.1 Introduction

Examining wild-caught samples only provides a limited insight into the mechanisms of evolutionary change. Although progressive, incremental, changes in the distribution of a population's phenotype over time may reflect genetic changes caused by natural selection while immediate, persistent, changes indicate a phenotypically plastic response to the new environment, there are alternative explanations. Incremental changes may reflect corresponding, incremental, changes in the environment. Conversely, immediate changes may indicate either a bottleneck in the founding population, or perhaps very strong selection pressures that cull unfit individuals immediately in the first individuals or offspring. To more fully understand the mechanism of evolutionary change, and gain insight into whether changes are the result of heritable changes in a population's genetic structure, or are instead caused by an environmentally induced response in a phenotypically plastic feature, one method is to rear fish from both populations in a common environment (Huntingford 1982).

Many of the morphological changes I observed in wild-caught Mayer Pond fish occurred immediately in response to the new environment. Spine and gill raker length, in particular, showed a significant and persistent reduction that occurred in the first generations after transplant. This may be the result of very strong selection on the transplant fish or on the first generation of their offspring, with only those with reduced spines and gill rakers surviving and breeding, or it could represent phenotypic plasticity, with some aspect of the environment affecting these traits. The loss of the anterior lateral plates in Mayer Pond fish seems to illustrate a different mechanism, with a progressive, incremental, change over time potentially indicating genetic changes by natural selection.

The fish collected as eggs and reared under identical conditions for the behavioural experiments discussed in Chapter 5 also provide a partial means to evaluate to what extent morphological changes observed in wild fish are the result of environmental influences or heritable components. If lab-raised Mayer Pond fish have significantly shorter spines and gill

rakers than Mayer Lake fish, when both groups have been reared under identical conditions of water chemistry and diet, then I will infer these features have a strong heritable component, and wild-caught differences therefore resulted from strong selection on the original transplanted fish or their offspring. If, on the other hand, there are no differences between Mayer Lake and Mayer Pond fish, then I will take this as evidence that the feature is phenotypically plastic, and is modified by some environmental cue(s). Similarly, the proportion of lab-raised fish with anterior lateral plates from the two populations will indicate how much this feature is influenced by the environment.

Examining the differences between clutches can also provide insight into the heritable components of a morphological feature. The stronger the heritable component of a feature, the more likely I expect to be able to distinguish between separate clutches. If a feature is entirely environmentally influenced, then when fish are raised under identical conditions there should be no differences between any of the clutches even from within a single lake. If, however, clutches can be statistically separated, this is evidence of heritable differences from the different parentages of each clutch. Furthermore, the one Mayer Pond clutch I was successful in rearing was split into two groups early on in development. If there are differences between these theoretically genetically equivalent groups, then this will indicate environmental differences in theoretically identical laboratory rearing conditions

4.2 Materials and Methods

Five lab-raised clutches of fish from Mayer Lake, and one clutch from Mayer Pond, are included in this morphological analysis (total n= 105, Table 8). The details of collecting and rearing lab-raised fish are described in Chapter 2. Once behavioural experiments (Chapter 5) were complete and the fish were sufficiently large (average size: 68 mm sl), individuals were euthanized with an overdose of Tricainemethanesulfonate (MS-222 or TMS) according to the University of Victoria Aquatic Unit Facility Standard Operating Procedure OA2003 for euthanizing fish. Specimens were preserved in 70% ethanol and photography, gill raker removal and staining, and morphological measurements from specimens and photographs were carried out as described in the methodology section of Chapter 3. Measurements of continuous traits were also size standardized as described in Chapter 3.

Table 8: Breakdown of lab-raised fish. Sample sizes indicate number of males and females raised from five different Mayer Lake (ML) clutches, and one Mayer Pond (MP) clutch raised in two separate groups (MP2a and MP2b). The average standard lengths of each sample group in millimeters are provided parenthetically.

	ML1	ML2	ML3	ML4	ML5	MP2a	MP2b	**TOTAL**
Female	5 (74.28)	17 (68.17)	19 (69.02)	9 (72.7)	3 (79.1)	13 (66.98)	2 (67.0)	**68**
Male	0	6 (67.6)	9 (64.3)	8 (66.85)	0	7 (63.25)	7 (67.0)	**38**
TOTAL	**5**	**23**	**28**	**17**	**3**	**20**	**9**	**105**

The statistical analysis is similar to described in Chapter 3. For differences in plate proportions I used a general linear model with binomial error structure to examine differences in the presence of left lateral plate 2 among the different clutches. For the continuous measurement I used Tukey post-hoc comparisons to identify which groups could be statistically separated from each other. I was interested in seeing if: (1) the Mayer Lake clutches were statistically equivalent; (2) there were detectable differences between Mayer Lake fish and Mayer Pond fish; (3) the two Mayer Pond samples (one clutch separated into two groups) were statistically the same. I did not conduct a principal component analysis due to the small sample size of the individual clutches. Instead I focused my analysis on size-standardized values of features notably different in wild-caught fish – maximum depth, gill raker length, plate presence, spine length, and ascending process dimensions. Similar to Chapter 3 I also conducted the analysis separately for males and females, and graph the degree of sexual dimorphism for each clutch to identify the consistency in dimorphism between clutches, and evaluate potential differences in the degree of dimorphism between lab reared Mayer Lake and Mayer Pond fish. The only exception is for the lateral plate presence data, in which I pooled the sexes as males and females showed similar trends, and to compensate for small sample sizes.

4.3 Results

4.3.1 Maximum depth

There is some evidence of a heritable change in the maximum depth of Mayer Pond fish compared to Mayer Lake lab specimens when both are raised in the lab. Males in the MP2b clutch are significantly deeper bodied than fish from all Mayer Lake clutches (Tukey post hoc comparison, $p<0.001$), with the exception of the ML5 clutch ($p=0.13$) that only has 3 individuals (Figure 31). However, the MP2b males are also significantly deeper than the MP2a males ($p<0.001$). MP2a males are statistically equivalent to all Mayer Lake clutches ($p>0.9$). In females, the only differences are in the MP2a clutch, in which individuals are suggestively deeper bodied than two of the five Mayer Lake clutches, ML3 and ML4 ($p=0.08$ and $p=0.09$, respectively). Although MP2b males indicate a potential heritable difference in body depth between Mayer Lake and Mayer Pond, there is a lack of consistency with the other, genetically equivalent, MP2a clutch. Over all the samples, males are deeper bodied than females, and particularly in the MP2b sample (Figure 31).

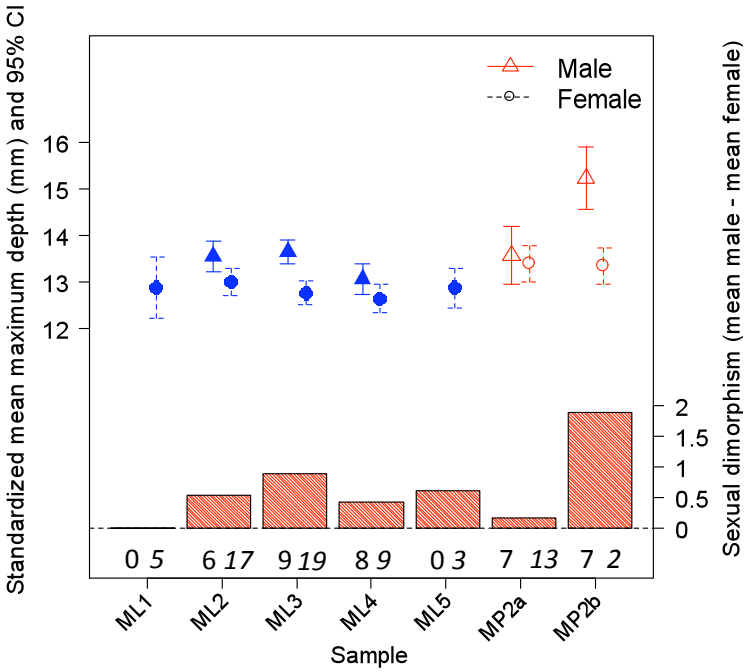


Figure 31: Means and 95% confidence intervals for the comparison of standardized maximum body depth (mm) for five lab-raised Mayer Lake (ML) clutches and one Mayer Pond (MP) clutch raised separately as two samples (MP2a and MP2b). Values have been standardized to those of a 60 mm fish. Males are represented with triangles and solid lines, females with circles and dotted lines. Mayer Lake clutches are represented by solid symbols, Mayer Pond samples are shown by open symbols. Numbers above each clutch represent the sample size for males and females. The bar graph at the base is described by the axis on the right hand side, and represents the degree of sexual dimorphism within each sample (mean male value – mean female value).

4.3.2 Gill raker length

There is some evidence of a heritable reduction when looking at gill raker 5 bone lengths, but only in females. In females, MP2a is significantly smaller than the ML3 and ML4 clutches (Tukey post hoc comparison, both $p < 0.03$) and suggestively smaller than ML05 ($p=0.08$). The MP2b individual female has an even lower trait value than the MP2a mean, but cannot be statistically compared as this datum has no variance. There is no difference between Mayer Lake and Mayer Pond for any of the male samples (Figure 32b). The highly correlated gill raker 3 bone length measurement (Pearson correlation =0.7, $p<0.001$) shows no differences between Mayer Lake and Mayer Pond fish.

In general, males seem to have larger gill rakers than females. Both Mayer Pond female samples show considerable smaller gill raker 5 bone lengths than males from the same sample. MP2b is not that reliable as there is only one female measurement, but the thirteen females in MP2a also show considerable dimorphism for this feature.

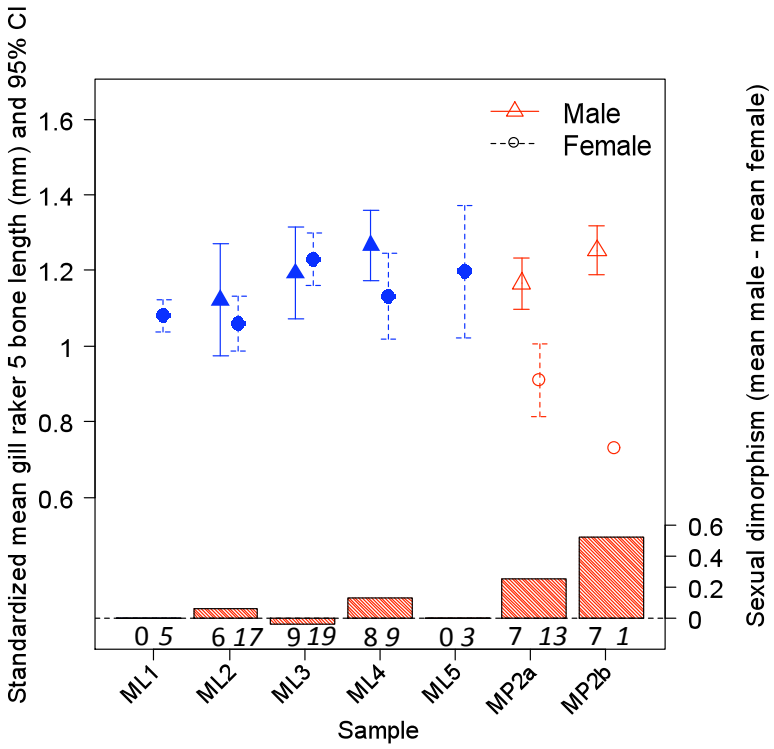


Figure 32: Means and 95% confidence intervals for the comparison of standardized gill raker 5 bone lengths (mm) in lab raised fish. (See Figure 31 for a detailed figure description.)

4.3.3 Plate presence

Overall, there is a considerable amount of variability in the proportion of individuals that have the llp2 between Mayer Lake clutches (ranging from 21% to 94% presence), and the overall plate presence is quite low (only 52% in Mayer Lake and 45% in Mayer Pond) in lab-raised fish. Granted, proportion data is potentially quite misleading given the small sample sizes of some of the clutches (e.g. if a clutch has only three fish and one is missing the plate, the plate presence is only 66% instead of 100%), so I grouped the sexes for this analysis since trends between the sexes are similar. But even in the largest clutch of pooled ML3 fish (28 individuals) the 2nd lateral plate presence is very low (21% presence). Mayer Pond fish cannot be differentiated from the Mayer Lake specimens with these lab-raised samples (general linear model with binomial error structure, $p>0.36$; Figure 33). Both groups also have an overall lower total left plate counts than their wild-caught counterparts (Mayer Lake: mean 6.95 plates in the wild, 6.56 plates in the lab; Mayer Pond: mean 6.8 plates in the wild, 6.07 plates in the lab).

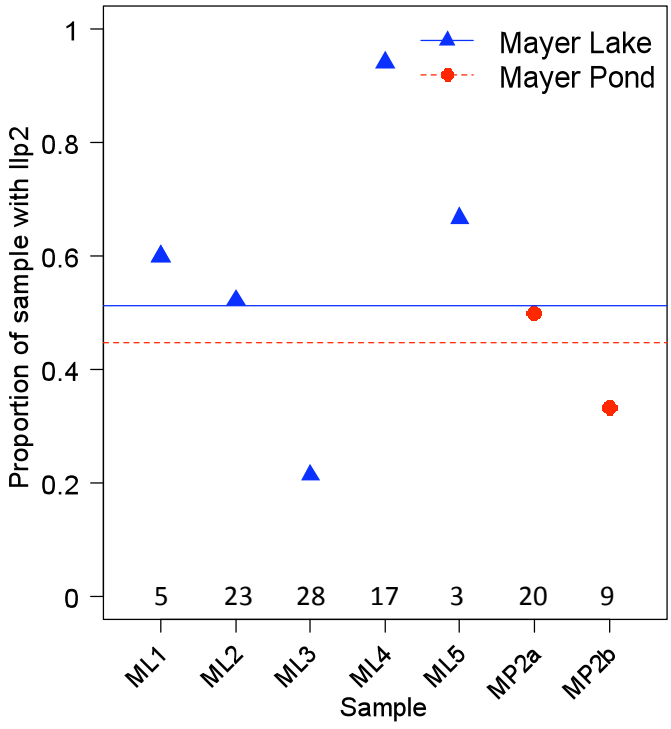


Figure 33: Proportion of five different Mayer Lake (ML, blue triangle symbols) and one Mayer Pond (MP, red circle symbols) clutches that have left lateral plate 2 (llp2). Numbers above clutch designations indicate sample size of each clutch. Males and females have been grouped together as the trends for each sex are similar and without grouping the sample sizes are quite low.

4.3.4 Spine length

Recall that in wild-caught comparisons there was an immediate and persistent reduction in dorsal spine length in Mayer Pond fish compared to Mayer Lake fish, and that females had larger spines than males in Mayer Pond samples.

There is some evidence of a difference in the second dorsal spine length between Mayer Pond and Mayer Lake fish. In males, MP2a and MP2b are significantly larger than ML3 (Tukey post hoc comparison, $p<0.01$ and $p=0.05$, respectively), and MP2a is almost significantly larger than ML2 ($p=0.08$). In females, both MP2a and MP2b are larger than the ML5 clutch (both $p<0.01$). However, the ML5 clutch is once again only three individuals, and it is also smaller than three of the other four Mayer Lake clutches (ML2, ML3 and ML4, all $p<0.001$). Other than a significant difference between the Mayer Pond samples and one Mayer Lake clutch for each sex, there are no additional differences between the second dorsal spine lengths of the two populations (Figure 34). The highly correlated first spine measurement (Pearson correlation = 0.87, $p<0.001$) shows similar trends, although there are even fewer differences between Mayer Lake and Mayer Pond lab-raised fish (only MP2a is different than ML5). Between the sexes, females consistently have longer spines than males.

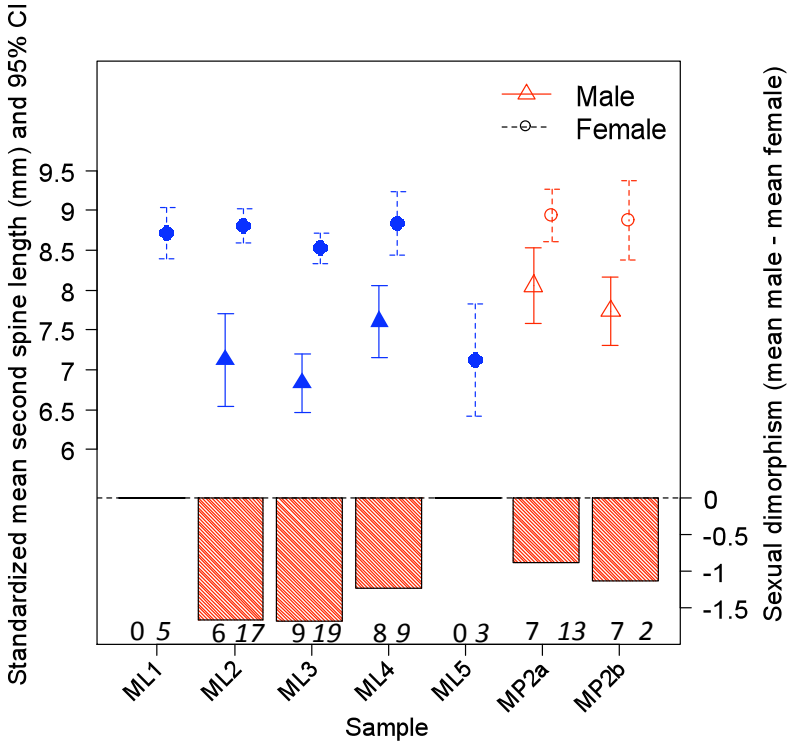


Figure 34: Standardized mean dorsal spine 2 length and 95% CI for lab raised fish. (See Figure 31 for a detailed figure description.)

There are no differences between the Mayer Pond samples and any of the Mayer Lake clutches for the left pelvic spine length measurement (Figure 35). However, like the dorsal spines just examined, females tend to have larger pelvic spines than males. If analyzed by sex, the first dorsal spine, second dorsal spine, and left pelvic spine, are all significantly larger in females compared to males (ANOVA with sex as a fixed factor, all $p<0.001$). The extent of this difference may be less in Mayer Pond fish compared to Mayer Lake samples (Figure 35).

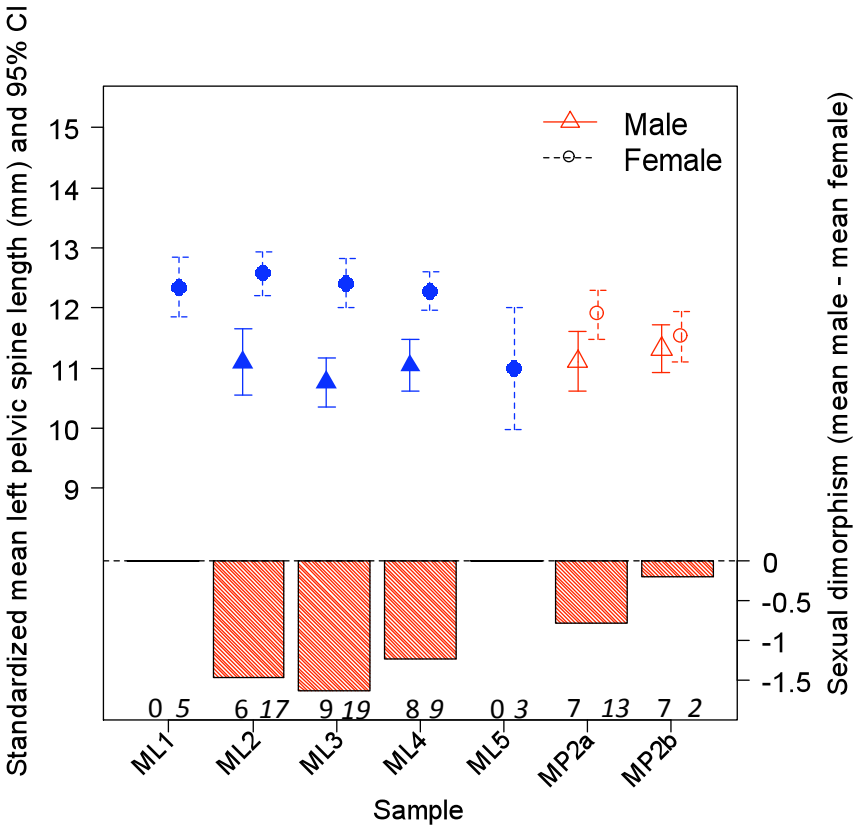


Figure 35: Standardized left pelvic spine length and 95% CI for lab raised fish. (See Figure 31 for a detailed figure description.)

4.3.5 Ascending process area

In wild-caught fish there was an immediate and persistent reduction of ascending process area in Mayer Pond samples compared to Mayer Lake individuals.

In lab-raised fish, there is no overall difference in the ascending process area of fish from the two populations, although there are a few differences between clutches (Figure 36). In males, both MP2a and MP2b are significantly larger than ML4 (Tukey post hoc comparison, $p<0.001$), but no other Mayer Lake clutch. In females there is only one difference between Mayer Pond and Mayer Lake, with MP2a being significantly smaller than ML2 ($p=0.05$). However, ML4 is also smaller than ML2 ($p=0.001$) and no different than MP2a ($p=0.81$).

Similar to wild-caught fish, males have larger ascending processes than females, and this dimorphism is greater in Mayer Pond samples than Mayer Lake fish (Figure 36).

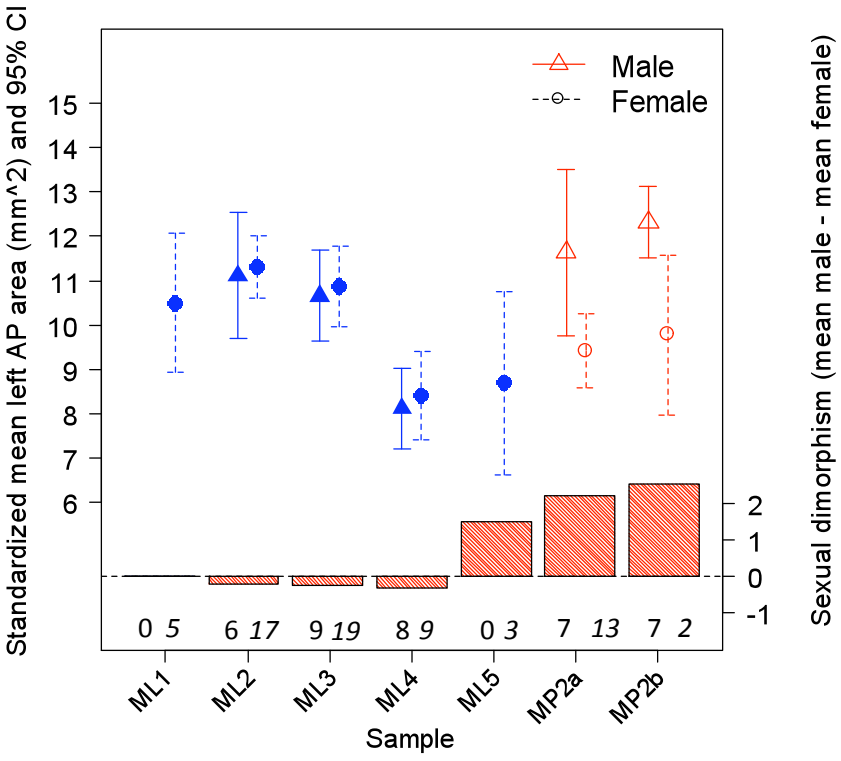


Figure 36: Standardized left ascending process area and 95% CI for lab raised fish. (See Figure 31 for a detailed figure description.)

4.4 Discussion

Experimental lab-raised populations provide the greatest opportunity for control in morphological studies (Bell 2001), and stickleback in particular lend themselves particularly well to rearing in the lab (Bell and Foster 1994). Combined with an interesting morphological diversity and the recent development of powerful genetic techniques (Peichel *et al.* 2001), a great amount of morphological research is carried out with lab-reared stickleback. My study is unique in that the populations under evaluation have a deliberate and known colonization history and, as seen in Chapter 3, many of the morphological changes appear to be environmentally induced. The lab-raised stickleback, although originally intended for behavioural experiments, provided an ideal opportunity to further evaluate the mechanism of morphological changes in Mayer Pond compared to Mayer Lake after 15 years since the original transplant of 100 fish from Mayer Lake.

The maximum body depth of wild-caught fish initially decreases following the transplant of fish from Mayer Lake to Mayer Pond, but by the most recent 2009 sample Mayer Pond individuals are significantly deeper bodied than Mayer Lake fish. A deeper body is the expected morphology given the relative habitat characteristics of Mayer Pond compared to Mayer Lake (smaller, no limnetic zone, extensive vegetation - see McPhail 1984, Schluter and McPhail 1992), and I hypothesized in Chapter 3 that perhaps the increasing body depth of Mayer Pond wild-caught fish was the result of selective pressures and heritable changes in the population. There is some evidence of a heritable increase in body depth from examining the lab-raised fish, with one Mayer Pond sample being significantly deeper bodied than all the Mayer Lake clutches. However, the other Mayer Pond sample, which should be genetically equivalent as it contains fish randomly separated from the same clutch, shows no difference. Given this lack of consistency between the two Mayer Pond samples from one clutch, and otherwise little difference between the Mayer Lake and Mayer Pond fish, there is no evidence from the lab-raised analysis to support my earlier hypothesis that the change in maximum depth represented heritable changes in the Mayer Pond population.

The gill rakers of wild-caught Mayer Pond fish undergo an immediate reduction compared to Mayer Lake fish, and there is some support that this is a phenotypically plastic response when looking at the lab-raised fish. In most cases there is no identifiable difference

between Mayer Lake and Mayer Pond lab-raised individuals. However, the 5th gill raker bone length is significantly shorter in the one Mayer Pond female sample compared to Mayer Lake fish. This measurement, at least in females, reflects a similar reduction in wild-caught fish and indicates that this reduction may be more heritable than originally inferred in Chapter 3. A high heritable component of gill raker length is not unique. Hagen (1973) concluded the heritability of gill rakers from lab-raised fish to be 0.58, Schluter (1996a) refers to a heritability of 0.44, and McPhail (1992) found that differences in gill raker length between populations bred true in lab-raised fish. However, although there is some evidence for heritable differences in this one measurement for this sex, otherwise there are no differences between Mayer Lake and Mayer Pond when raised in the lab on an identical diet. Day *et al.* (1994) similarly found that dietary differences, or lack thereof, were an important predictor in the plastic development of gill raker length in lab-raised sticklebacks. Some environmental difference between the two populations has therefore influenced the tremendous and immediate reductions seen in the wild-caught fish.

A reduction in the presence of llp2 in wild-caught Mayer Pond fish occurred progressively over time and appeared to represent heritable changes in the population, but we cannot support that inference as the lab-raised fish from both populations show no statistical differences. However, there is a tremendous amount of variability in llp2 presence between Mayer Lake clutches, and the presence of that plate is much lower in the lab-raised fish than wild-caught fish. Plates are developed by 28 mm (Hagen 1973), well below the size of these samples, and it is not possible that the fish had simply not developed the plates yet. Since the heritability of these plates is well documented in lab-raised fish (Hagen 1973, McPhail 1992), as are the genetic mechanisms controlling plate development (Peichel *et al.* 2001, Colosimo *et al.* 2004, Knecht *et al.* 2007), I don't think this low llp2 presence is the result of environmental influences from the lab. Instead there are a few explanations: 1) Perhaps this variability (from 16%-100%) is present in the wild, but the low llp2 individuals do not survive to the point of our sampling of adults due to the documented importance of the anterior lateral plates in protecting against puncture wounds from fish predators (Reimchen 1992). However, it is odd that these llp2 absent individuals would continue to represent much of the population with this strong selective pressure. 2) Perhaps, with only five clutches, we happened by chance to collect eggs from parents without llp2 – individuals uncommon, but still present, in Mayer Lake. 3) There has been a strong selective pressure in the laboratory, in the opposite direction as in the

wild, which increased the fitness of llp2 absent individuals compared to those with more plates. During the rearing of the fish we had technical difficulties which resulted in massive swings in pH and killed 90% of the lab-raised fish. If this mortality was non-random, and individuals with fewer plates and specifically fish without llp2 were better able to survive this 'artificial selection', than this otherwise uncommon segment of the intraclutch variability would have become much more dominant. Giles (1983b) postulated calcium availability as a selective force affecting plate frequencies, and (Barrett *et al.* 2008) found individuals with fewer plates grew faster when salinity was lower, but as far as I know there has been no research about how pH and resulting physiological stress/mortality could be influenced by plate presence in threespine stickleback.

The remaining features that showed a change in wild-caught fish, spine lengths and ascending process area, appeared from the wild-caught samples to be an immediate and environmentally induced response and this interpretation is supported from examinations of lab-raised fish. The lengths of dorsal spines, the pelvic spine, and the area of the ascending process are, for the most part, indistinguishable between Mayer Pond and Mayer Lake lab-raised individuals. Although with the spine measurements there is one Mayer Lake clutch that is statistically different from Mayer Pond, not only are the differences not replicated in the other Mayer Lake clutches, but the direction of difference (longer spines in Mayer Pond fish) are opposite to those found in wild-caught fish where Mayer Lake fish have significantly longer spines. Therefore these data from lab-raised individuals do not refute my original interpretation in chapter 3 that the reduction in spine length of Mayer Pond fish is the result of phenotypic plasticity, a finding in contrast to the relatively high heritability put forth by other studies (McPhail 1992, Hatfield 1997, Peichel *et al.* 2001).

Although there were no differences between fish from the two lakes, there was a consistent sexual dimorphism in the lab-raised fish as in the wild-caught fish. Males have shorter spines, larger ascending processes, and potentially longer gill rakers, than females in both the lab-raised and wild-caught specimens. Most of these features have been not been investigated with respect to sexual dimorphism, except for spine length. Although a widespread geographical investigation of the correlation of ecology and morphology in threespine stickleback found that males typically had longer spines than females (Reimchen and Nosil 2006), an opposite trend to what I have found in both the wild and lab-raised fish, in other situations where predatory fish are absent, similar to Mayer Pond, males often have shorter spines (Reimchen 1980, Reimchen

and Nosil 2004). The smaller spine length in males compared to females, especially in Mayer Pond, I observed is therefore consistent with other ecologically similar situations.

Overall, my conclusions from examining the rate of change in the wild-caught populations have been supported by looking at these populations raised in identical conditions in the laboratory. There is some evidence of a heritable component to gill raker length which I did not expect, but in general the evidence is in favour of a primarily environmental influence changing the length of this feature in the wild. Other features I previously inferred to be plastic – spine length and ascending process area – are mostly equivalent when fish are raised under identical conditions. Of note is the apparently heritable sexual dimorphism in spine length, ascending process area, and possibly gill raker length. Also interesting is the amount of variability and low presence of llp2 in the Mayer Lake lab-raised clutches. This feature is quite conserved in the wild Mayer Lake samples, and perhaps this reduction points to a period of artificial selection from massive pH changes and resulting high mortalities in lab-raised fish. The heritability of llp2 indicated by a progressive change in successive generations since transplantation in wild-caught fish cannot therefore be evaluated in these lab-raised individuals.

When considering the morphological features that showed changes in wild-caught samples, overall I failed to find any consistent evidence for differences between Mayer Lake and Mayer Pond fish when examining lab reared individuals (Table 9). While this supports my inferences of phenotypic plasticity for the features that underwent an immediate and persistent change following transplant in the wild specimens, it is concerning that I did not find any of the expected differences indicative of heritable changes (i.e. for maximum depth or left lateral plate 2 presence). This may be a reflection of an inability to detect differences with these samples, rather than an actual lack of differences. These potentially heritable differences in lab-raised fish may be obscured due to: (1) the small sample size (and hence higher variability) of some of the samples; (2) a lack of heritable differences for these features in these populations; (3) the result of only having one genetic Mayer Pond clutch with which to compare. Additional lab-raised specimens from both Mayer Lake and Mayer Pond are needed to more fully evaluate the phenotypic plasticity vs. heritable changes for features that are different between Mayer Lake and Mayer Pond sticklebacks, as well as determine which environmental factors drive phenotypically plastic changes.

Table 9: Summary table of morphological comparisons of Mayer Pond fish to Mayer Lake individuals when examining wild-caught specimens following an initial transplant of fish from Mayer Pond to Mayer Lake (see Chapter 3) and between those two populations using fish reared entirely in the laboratory.

Feature	Wild-caught fish	Lab-raised fish
Maximum depth	Initial decrease, but then steady increase until significantly larger than Mayer Lake fish in most recent sample	Larger in one Mayer Pond sample, but not the other from the same clutch.
Gill raker length	Immediate and persistent decrease following transplant	Decrease in females, but not in males.
Anterior lateral plate presence	Progressive decline from over 90% presence following transplant to 60% presence in most recent samples	No difference between Mayer Lake and Mayer Pond individuals, but both populations have a lower anterior plate presences and overall plate counts compared to wild counterparts.
Spine length	Immediate and persistent decrease following transplant	No consistent difference between the two populations. Only potential change is in opposite direction, with Mayer Pond fish having longer spines than Mayer Lake specimens.
Ascending process area	Immediate and persistent decrease following transplant	No difference between Mayer Lake and Mayer Pond.

Chapter 5: Evaluating the behavioural responses of two stickleback populations from fundamentally different habitats using lab-raised individuals

5.1 Introduction

The threespine stickleback is a model species for behavioural studies (Bell 1995b), and has a number of unique and complicated behaviours, such as the mating displays noted by Tinbergen (1952). Differences in behavioural responses of sticklebacks to predators between populations subjected to different forms and intensities of predation have been studied for over 25 years (some of the first examples include: Huntingford 1982, Giles and Huntingford 1984). However, since those studies it has been recognized that lab-raised individuals are necessary to identify whether behaviours are the result of different experiences between habitats, or some heritable component of behaviour driven by differences in selective pressures between the habitats (Huntingford 1982). Also, while there is as well as growing evidence that experience with predators, even at a young age, modifies subsequent behaviour (Bell and Sih 2007), there have been few studies, other than a few exceptions (seeBell 2005, Dingemanse *et al.* 2009), that have used entirely lab-raised fish (for recent examples see: Alvarez and Bell 2007, Messler *et al.* 2007, Brydges *et al.* 2008b).

In 1993 100 threespine sticklebacks were transferred from Mayer Lake to Mayer Pond, a fundamentally different habitat. As described in Chapters 1 and 2, these two habitats differ in a variety of features – size, water clarity, vegetation, prey type, and predatory pressures. The last, predation pressure, has been particularly well studied in other stickleback populations and has been linked to differences in a number of behavioural characteristics such as boldness and aggression (Huntingford 1982, Dingemanse *et al.* 2007, Brydges *et al.* 2008a), as well as more complex responses such as willingness to explore novel environments (Dingemanse *et al.* 2007), and even high level learning abilities and spatial recognition (Brown and Braithwaite 2004, Brydges *et al.* 2008b). Given that predation pressure is a major predictor of behavioural differences between populations (Bell and Stamps 2004, Dingemanse *et al.* 2007), in addition to the other ecological differences between these two populations, I predict there are behavioural differences between Mayer Pond fish and the ancestral Mayer Lake population.

Specifically, I predict that there will be differences that reflect the predators of the two habitats. The primary predators in Mayer Lake are diving birds and larger species of fish (e.g. cutthroat trout). Mayer Pond has neither type of predator, but instead stickleback may be eaten when young (less than 30 mm) by macroinvertebrate larvae such as *Aeshna* larvae (Reimchen 1980). Adult stickleback may also prey on young fry (less than 15mm) in both habitats (Foster *et al.* 1988). The difference between the physical characteristics and hunting strategy of gape limited pursuit-orientated vertebrate predators versus grasping, ambush invertebrate predators considerably affects defense morphology (Reimchen 1980, 1994) and presumably behaviour as well. I expect that Mayer Lake fish will be more sensitive to overhead movement representing an attack from a diving bird than Mayer Pond, and I predict maximum antipredator behaviour in response to habitat appropriate olfactory cues – trout for Mayer Lake fish, dragonfly larvae for Mayer Pond fish.

Although I predict that there are behavioural differences between Mayer Lake and Mayer Pond populations, differences between populations could be caused simply by divergent experiences if the fish are not raised entirely in the lab. Since fish have a J-shaped mortality curve (the majority of mortality is at the earliest life stages) the strongest selective pressures occur early in development. The resulting experiences shape the future behaviour of even very young individuals. For example, stickleback less than three weeks old show the beginnings of predator inspection behaviour and modify their responses based on the perceived relative difference in size between them and a predator (Bishop and Brown 1992), and predation by conspecific adults on fish less than 15 mm modifies habitat use by young fry (Foster *et al.* 1988). As I am interested not only in whether there are differences between the behaviour of Mayer Lake and Mayer Pond fish, but also the mechanism of any change (i.e. are differences the result of heritable, genetic changes, or environmentally induced plasticity), I raised fish from these two populations collected as eggs and reared under identical conditions in the laboratory.

I reared five clutches of eggs from Mayer Lake and one clutch from Mayer Pond in the laboratory at UVic and conducted trials over the course of the fishes' development. From one to 10 months of age I tested for differences in simple preference trials, and response to a variety of predatory cues that would be detected by different sensory systems including olfactory, visual, and pressure stimuli, to identify any heritable, behavioural differences between Mayer Lake and Mayer Pond.

5.2 Methods

Details of egg collection and rearing are described in Chapter 2.

5.2.1 Substrate and cover preference experiments

When the fish were approximately one month old (approximately 10mm) I conducted simple substrate and cover preference experiments. Both experimental types were conducted using glass watch glasses bounded below and from the sides by white matboard (Figure 37). I had six such containers and tested six fish (three from Mayer Lake and three from Mayer Pond) each trial (total of 20 trials for each type of setup). I changed the water between trials and alternated the positioning of Mayer Lake and Mayer Pond to eliminate unanticipated environmental effects. Trials lasted two minutes and were scored every thirty seconds (with remotely taken still pictures or video taken from above).

Substrate preference experiments involved placing a checkerboard pattern of black and white under the watch glass and noting the position and activity of the fish every thirty seconds (Figure 37 and Figure 38a). Every thirty seconds the substrate preference of the fish was determined by whether their head was over a black or white section, and the fish was classified as active for the previous time interval if they had changed position (even within a section) during the previous 30 seconds. I carried out substrate preference experiments on two separate days (two week separation) and tested 30 fish from each lake on each day (total n=120). I sampled, with replacement, from the largest clutches: Mayer Lake 1 clutch (approximately 268 fish) and Mayer Pond 2 clutch (approximately 322 fish). I analyzed the data by examining the proportion of the fish (out of 60) for each lake that were over white in each of the five time periods (0, 30, 60, 90, and 120 seconds) and the proportion of fish that were active in each of the four time intervals (0-30, 30-60, 60-90, and 90-120 seconds). I used a general linear model with binomial error structure (as appropriate for proportion data) and model simplification using AIC values to identify the best model (most explanatory power with fewest variables), to see if there were trends over the course of the trials, and if there were overall differences between the lakes. I also used a binomial test to identify if the proportion of fish over white and if the proportion of fish active were different between the two populations at each time interval.

The cover preference trials involved a uniformly white substrate and a white piece of laminated cardboard that covered half of glass dish (Figure 37 and Figure 38b). After the fish

were in the glass dish the cardboard was placed on top of the dish effectively providing 50% cover and 50% uncovered area. The trials occurred over two days (two weeks apart) and involved 30 fish from Mayer Lake and 30 fish from Mayer Pond each day (total n=120), sampled with replacement from the Mayer Lake 1 and Mayer Pond 2 clutches. Every thirty seconds I noted, from photographs taken from directly above, whether each fish was hidden beneath the cover or visible by being in the non-covered area. I again used a general linear model with binomial error structure and AIC model simplification process to identify if there were trends in the use of cover over the trial, and if there were differences in the proportion using cover between the lakes. I used a binomial test to identify if the proportion of fish under cover was different between the two populations at each 30 second time interval.

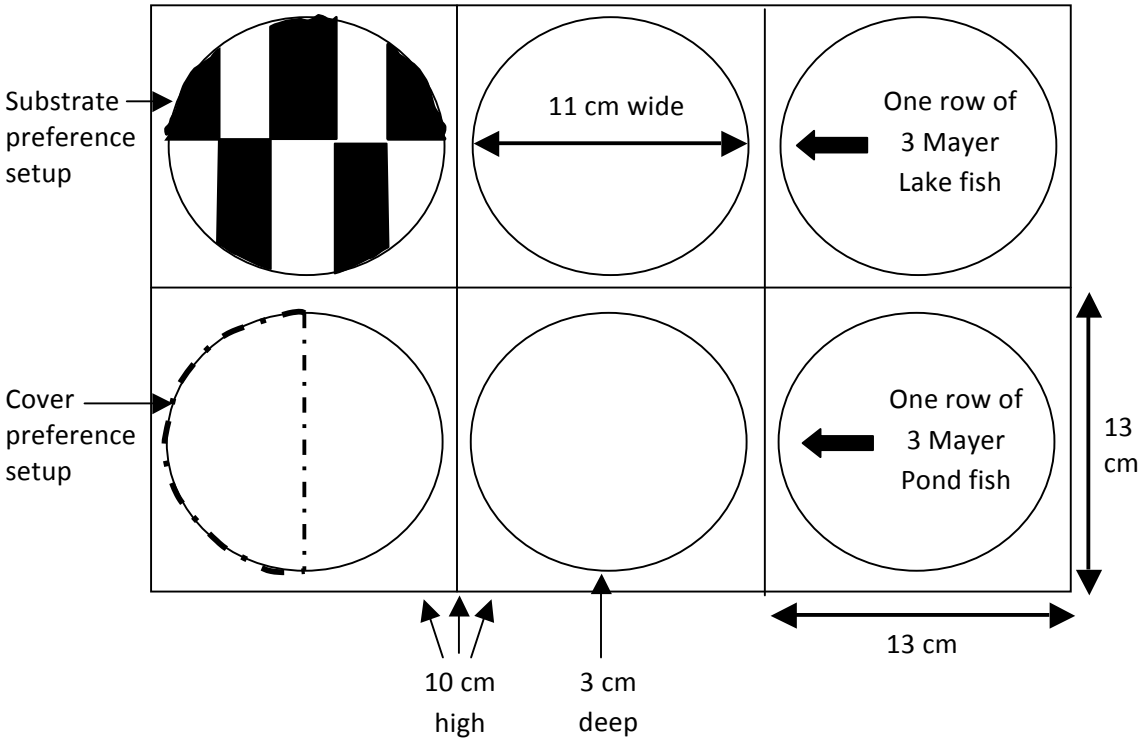
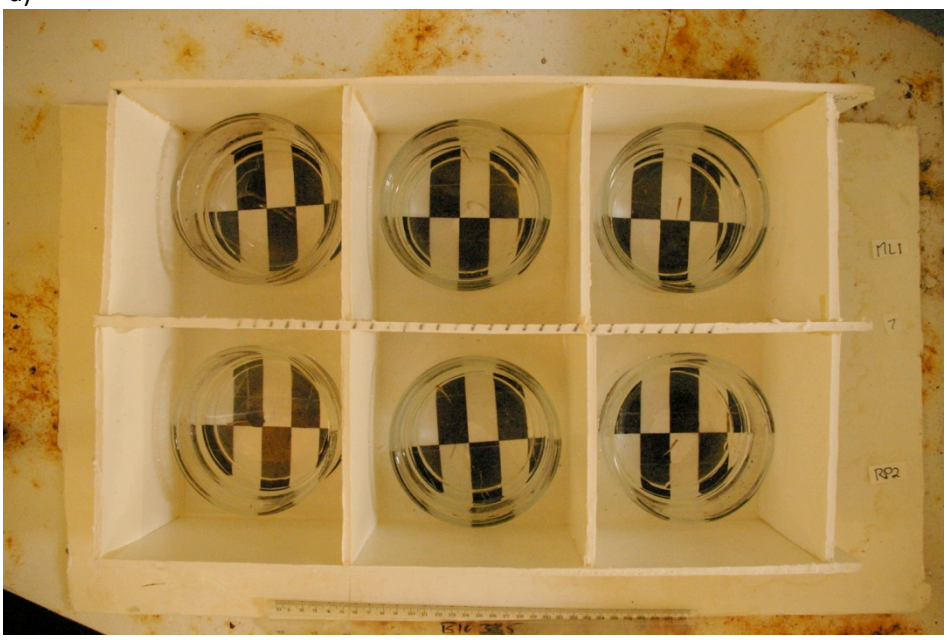


Figure 37: Preference experiment setup as seen looking down on from above showing both substrate and cover preference trial setups within 13 cm square white matboard walls. Six trials were run at once, with all dishes either having a black and white checkerboard substrate for substrate preference trials, or a half circle cover for cover preference trials. There were three fish from Mayer Lake in one row, and three from Mayer Pond in the other row. The dishes were rinsed and filled with fresh water for each trial and the positions of Mayer Lake and Mayer Pond individuals was alternated each trial.

a)



b)

Figure 38: Example photo taken for a) substrate preference trials, and b) cover preference trials. These photos were used for scoring the fishes presence and movement in these two types of observational experiments.

5.2.2 Olfactory cue experiments

I conducted olfactory cue experiments when the fish were about 2.5 months old (approximately 20 mm) to examine any differences between Mayer Lake and Mayer Pond in their response to predatory olfactory cues. I tested a total of 53 fish and three different predatory cues: (1) A living dragonfly (Genus: *Aeshna*) larvae (14 Mayer Lake fish, nine Mayer Pond fish); (2) Cotton skin swabs from the flank of a freshly killed rainbow trout (*Oncorhynchus mykiss*; eight fish from each lake); and (3) The dragonfly larvae with a decapitated stickleback that had recently died of natural causes (seven fish from each lake). The dragonfly larvae used in this experiment was collected locally, maintained with white worms, and fed stickleback fry that had died of natural causes whenever possible to maximize predatory olfactory cues exuded from this organism (I attempted to magnify this effect with the inclusion of a freshly decapitated fry in the last experimental trials). Cutthroat trout (*O. clarkii*), a primary predator in Mayer Lake, were not available, but rainbow trout, a closely related salmonid, were kept at the aquatic facility and periodically culled. Skin swabs from a freshly killed specimen were the closest I could come for olfactory cues from this potential predatory group.

The experiments were set up the same way regardless of the predatory olfactory stimulus. Within a box made of white corrugated plastic and having a removable lid I placed a 20 cm Y-maze made of a plastic tray, Styrofoam walls, and hot glue. Water flowed into each arm of the Y and out through small perforations at the end of the chamber. The water flowing into each arm of the Y first passed through a 500 mL container placed outside the testing chamber. One 500 mL container was only filled with water providing a freshwater control, while the other had one of the three predatory stimuli (dragonfly larvae, three trout skin swabs, or dragonfly larvae and decapitated fry). Adjustable clamps ensured that water flowed through these containers and into each arm of the Y-maze at an equivalent rate of 80 mL/min. Halfway down the length of the Y-maze was a plastic gate which, when present, restricted the fish's movement to the back part of the chamber, but could be removed by pulling a string led outside the chamber (Figure 39).

Trials then proceeded as follows. First, the arm that was to be the control was randomly decided (this was switched half way through a day's trials so that there was no side bias). An individual fish was then placed in the chamber behind the gate, the lid was placed on, the gate was removed and fish were allowed to swim around the Y maze. Trials were allowed to proceed

for two minutes after the fish crossed the gate line (where the gate had been inserted) or for a total of five minutes if the fish never left the back part of the Y. I alternated Mayer Lake and Mayer Pond individuals and between trials I rinsed the container with fresh water. After testing individuals were kept separately so they could not be tested more than once on a given experimental day. The base of the Y maze was divided into sections (see Figure 39) and trials were scored for the following:

1) Time to cross – the time from when the gate is removed to when the fish crosses the gate line
2) Events per minute – number of times a fish crossed from one section into another, or made a 180 degree change in direction within a section, divided by the length of the trial in minutes
3) Proportion of time spent in sections opposite to the predatory stimulus.

All data were analyzed with an ANOVA including lake and predatory stimulus as factors to identify whether there was a difference in the response between Mayer Lake and Mayer Pond fish, between the different predatory stimuli, and any potential interactions between the two.

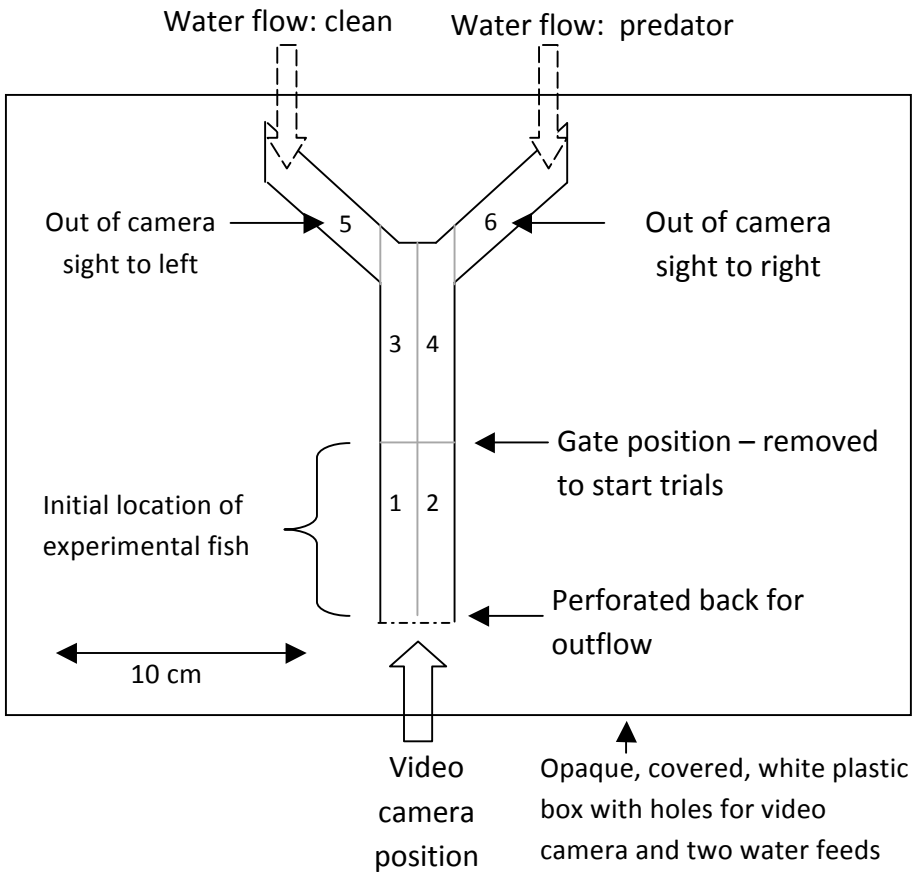


Figure 39: Overhead view of the experimental setup of Y maze for olfactory trials. Sections numbered 1 through 6 were used for scoring activity level of subjects – movement from one section to another, or a dominant change in direction within a section, were classified as 'events'. I tested three different predator cues - (1) A living dragonfly (Genus: *Aeshna*) larvae; (2) Cotton skin swabs from the flank of a freshly killed rainbow trout (*Oncorhynchus mykiss*) and (3) The dragonfly larvae with a decapitated stickleback that had recently died of natural causes – and scored the fishes' behaviour using video taken through a small hole at the back of the container.

5.2.3 Overhead movement and pressure burst predatory cue experiments

When the fish were approximately six to 10 months old I conducted experiments where I presented a variety of simple predatory cues. These cues targeted different sensory modalities and included: 1) Movement overhead (visual); 2) Burst of water (pressure); and 3) Trials conducted in water from a trout tank (olfactory). I tested these cues alone or in combination, and for some cues I ran tests both in diffused light and completely dark conditions. The predatory stimuli used were chosen as they represent basic sensory stimuli that may be presented by predators in one or both of the two populations. Dark conditions were included as Mayer Lake is a black water environment with light attenuation by 2 m in depth (Reimchen 1989) while Mayer Pond is brightly lit.

All experiments took place with the same basic setup (Figure 40). Fish were placed into a 15 cm cubed plexiglass tank filled with 10 cm of fresh system water. This tank was enclosed within a larger container that was made of opaque black corrugated plastic and which prevented external visual stimuli from all directions other than above. However, in trials conducted during the day a semi-transparent piece of white corrugated plastic was placed overhead and lit with a 60 Watt bulb providing a relatively uniform diffused light ceiling that blocked external visual stimuli from above. During the night trials the facility was pitch black and other visual stimuli were not a concern. (Note: for safety reasons I briefly used a dim red darkroom light placed some distance from the tanks that provided sufficient illumination to catch and move the stickleback. This light was turned off during trials.) For the night trials, specialized LEDs placed in a modified flashlight provided illumination at wavelengths above 880 nm which is beyond the spectral sensitivity of stickleback or human eyes (Boulcott and Braithwaite 2005), but quite visible on the video camera. All trials were videotaped from the side at the same height as the tank through a hole cut in the black corrugated plastic. A curtain of opaque material was placed around the camera lens to ensure no movement or shadows could be seen by the experimental fish. Individual fish were given two minutes to acclimatize to the tank and then were presented with a predatory stimulus.

To provide a visual stimulus, a black rectangle made of lego (5 centimeters by 8 cm) passed over the tank at a relatively constant rate of approximately 5 cm/sec. This object was suspended on clear fishing line 10cm above the water level of the tank and moved by a system of counterweights and pulleys. It started and stopped outside of the fish's field of view (which was previously identified using Snell's law and the height of the water) and was released by

detaching a clamp positioned outside the experimental chamber. The movement of this dark shape, especially as it was backlit with the bright, diffusely lit, 'ceiling' was intended to mimic the shadow of an overhead predator such as a bird.

A small burst of water provided a pressure stimulus. This was created with a piece of thin plastic tubing that was attached at one end to an elbow junction glued in the middle of the inside of the tank, fed outside the experimental chamber, and connected at the other end to a small pipette bulb. Before trials the bulb was pumped a few times filling the tube with a few mL of water. At the appropriate two minutes a spring loaded clamp was allowed to close on the pipette bulb. This provided a horizontal burst of water that was of consistent volume and rate across trials. This burst was intended to mimic the horizontal pressure wave preceding an approaching predator. For pressure and visual stimuli combined trials, which not only increased the intensity of stimulation, but also better represented the combination of stimuli that may precede an attack, I released the pressure burst as the visual stimulus came into the fish's field of view.

Olfactory cues were provided by using water from a high density Rainbow Trout tank. These trials were not conducted in isolation, but were done in combination with visual and/or pressure stimulus trials to magnify the predatory stimuli and, potentially, the experimental fish's resulting antipredator response. The trout water was of equivalent pH and temperature as the normal system water used in all other trials. Fresh trout water was used for each trial and the plexiglass chamber was rinsed with trout water between trials. In non-olfactory trials fresh system water was used in each trial and the tank was rinsed with normal system water between trials to prevent confounding cues as stickleback have demonstrated conspecific olfactory signals (Rafferty and Boughman 2006).

Trials conducted in light and dark conditions proceeded as follows. I filled the plexiglass tank with the appropriate water source (fresh system or trout water), captured an individual Mayer Lake or Mayer Pond fish, and placed it in the tank. I covered the chamber with the semi-transparent plastic and light if during the day, nothing if at night, and allowed the fish two minutes to acclimatize. After two minutes I released the appropriate stimulus (alone or in combination for visual and pressure stimuli) and continued filming for an additional two minutes. I then removed the fish to separate holding tank, rinsed the plexiglass container and set up for the next trial. Tested fish were kept separated from their clutch on a given experimental day so they could not be tested twice on a given day. At the end of a day's trials

they were returned. I alternated testing Mayer Lake and Mayer Pond fish on most days. However, as there were more clutches and individuals from Mayer Lake on some days I only compared different Mayer Lake clutches. All clutches were tested with all experimental setups (see Table 10 for a complete breakdown of experimental trials by date, type and clutches tested).

Table 10: The clutch and number of fish tested over 4 months of trials. Different experiment type codes include: v=visual stimulus, p=pressure stimulus, l=light, d=dark, c=clean water, t=trout water, v+p=visual and pressure stimulus. Italicized rows are days were only ML clutches were compared. Numbers in brackets next to clutch represent approximate clutch size at time of the experiments. Fish were tested without replacement on a given date. Note: one visual trial was conducted at night to ensure that 1) no additional cues were presented by the stimulus, and 2) that night trials were completely dark. As there was no response to the visual stimulus in night trials it was excluded from any additional dark trials. Additionally, only a maximum of two stimuli were presented at once (hence why there are no v+p trials in trout water).

		Clutch						
Date	Experiment	ML1 (11)	ML2 (28)	ML3 (33)	ML4 (23)	ML5 (7)	MP2a (23)	MP2b (9)
21/12/07	v_l_c			3				3
24/12/07	v_l_c			8	7		8	7
8/1/08	*v_l_c*	*7*	*7*			*6*		
29/1/08	v_d_c			7				7
30/1/08	v_d_c				7		8	
4/2/08	*v_d_c*	*7*						
5/2/08	*v_d_c*		*5*			*5*		
1/3/08	p_l_c	7			7		7	
11/3/08	p_l_c		7	7		7		7
16/3/08	p_d_c	7			7		7	
17/3/08	p_d_c		7	7		7		7
11/4/08	v+p_l_c			7	7		7	7
14/4/08	*v+p_l_c*	*7*	*7*			*7*		
15/4/08	v_l_t			7	7		7	7
16/4/08	*v_l_t*	*7*	*7*			*7*		
17/4/08	p_l_t			7	7		7	7
18/4/08	*p_l_t*	*7*	*6*			*8*		
21/4/08	p_d_t			7	7		7	7
22/4/08	*p_d_t*	*7*	*3*			*3*		

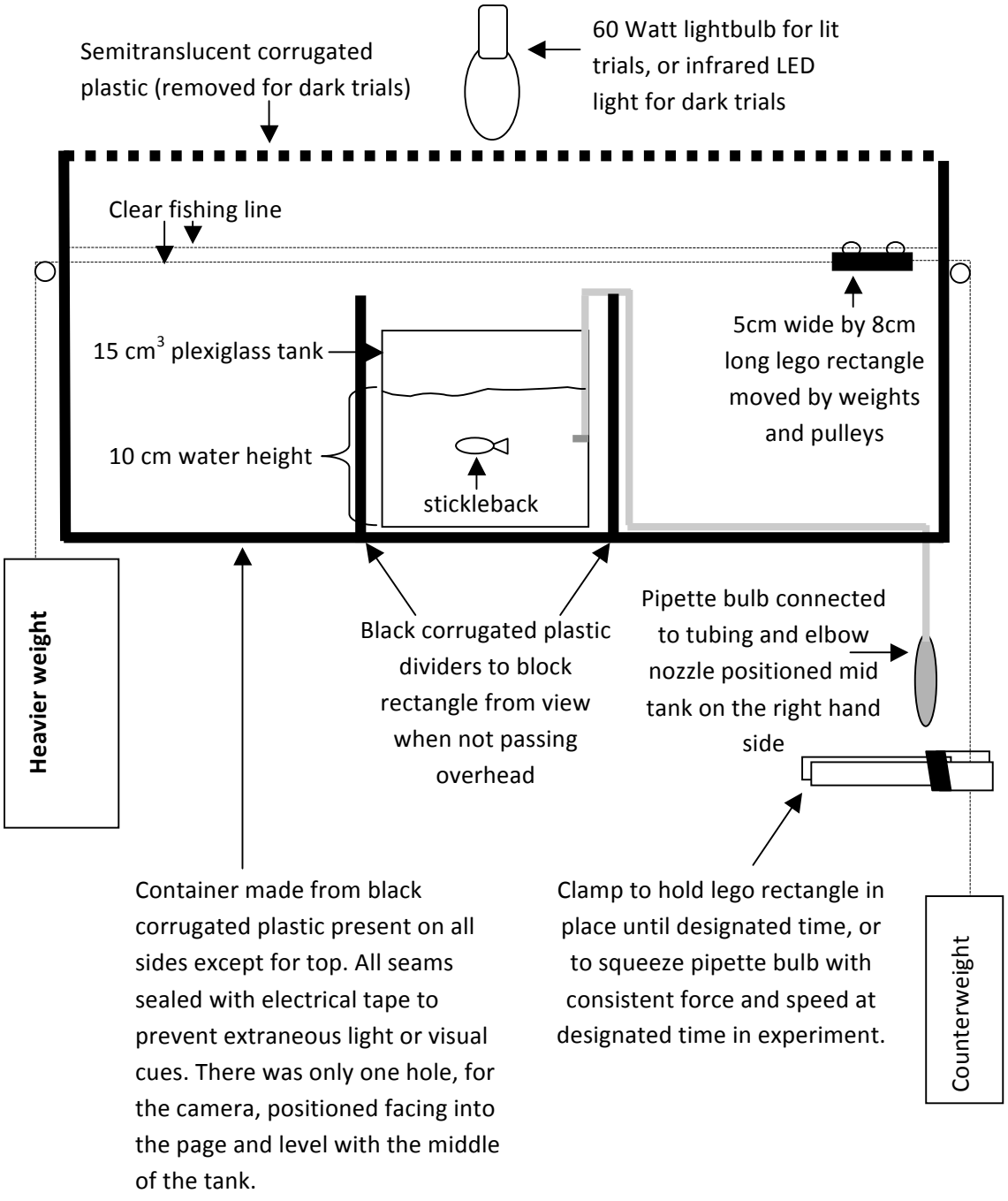


Figure 40: Setup for predatory stimuli trials with variable light conditions (lit or dark), water conditions (normal or trout), and predatory stimuli (overhead movement from the black rectangle, pressure burst from the pipette bulb, and the two combined).

Video footage from the trials was transferred to DVD and the fish's behavior was scored for the following features as similar measures have found to have been informative in other antipredatory behaviour assessments in stickleback (Tulley and Huntingford 1986, Huntingford and Giles 1987).

- **'Pre' movement:** A 30 second window, five seconds before the stimulus is released, in which I noted the activity of the fish. This was quantified by superimposing a 12 square grid over the video of the tank and counting how many squares the eye of the fish passed through during this time. (Even if the fish did not move it got a value of 1).
- **'Pre' spines**: The general state of the spines during this 30 second 'pre' window. Originally classified as: 1: spines never to rarely raised; 2: spines occasionally raised; 3: spines often raised when stopped; 4: spines almost always ways raised, moving or not, but then data were re-categorized and analysed as 1: spines not raised (categories 1 and 2); and 2: Spines often up (categories 3 and 4) for improved consistency in a potentially subjective measure.
- **Reaction to the stimulus**: Defined as 1) no reaction; 2) investigation, rising or following the direction of stimulus; 3) cessation of normal movement – freezing, or sinking, or slowly moving away; 4) rapid escape burst; 5: slow escape burst. These were grouped more generally into: 1) no adverse reaction (previous categories 1 and 2); 2) cessation of normal movement (previous category 3); 3) escape burst (previous categories 4 and 5).
- **Spine response:** State of the spines (raised or lowered) in response to the stimulus.
- **Recovery time**: The length of time after the stimulus ended before normal, pre-stimulus, movement started again (default of 0 if no reaction to stimulus).

Because individuals developed over the six months that these trials took place, the analysis of these data was restricted to comparisons within, rather than across, experimental setups. Changes in the developmental stage of fish (including potential reproductive status) affect the behaviour of stickleback (Rodewald and Foster 1998) and, combined with fluctuations in predatory pressure that change temporally and seasonally in the fishes' native habitat (Reimchen 1994, Reimchen and Nosil 2002), this complicates comparisons of behaviour across

six months. Furthermore, the fish were used repeatedly over the course of the trials. Analyzing data over the entire course of the experiments constitutes pseudoreplication, a statistical violation. Additionally, given the different clutch sizes and variable likelihood of being used in previous experiments (e.g. every Mayer Lake 5 fish was used in each experiment, whereas a member of the Mayer Lake 3 clutch had only a 21% chance of being chosen for a given trial), the importance of early predatory experience shaping behaviour (Kelley and Magurran 2003), and stickleback's demonstrated learning capacity (Brydges *et al.* 2008b), it is inappropriate to use conventional statistical methods to analyze data pooled over the six months of trials.

Fortunately, I was most interested in whether Mayer Lake fish differ in their behavior compared to Mayer Pond individuals when raised in the lab, rather than whether, in general, fish respond differently to an overhead stimulus versus a pressure burst. The different stimuli simply provided repeated opportunities and means to identify potential differences between these two populations. Therefore, I compared the response of the five Mayer Lake clutches, and one Mayer Pond clutch separated into two groups, within each experimental type. Differences present only in one type of experiment are informative as to what predatory cues each population is picking up on, and any trends that are replicated and consistent across experiments provide added support for behavioural differences between the two populations.

Within each type of response variable, I analyzed the difference between Mayer Lake and Mayer Pond fish for each experimental setup using statistics appropriate for those data. For pre movement, which was count data, I used a general linear model with Poisson error structure. For the pre spine position, overall response, and spine reaction to the stimulus, which were all categorical data, I used a chi-square test looking at the dependence of response on source locality of fish (Mayer Lake vs. Mayer Pond). For the recovery time I used a t-test to see if the activity change was significantly different between Mayer Lake and Mayer Pond fish. I could test differences in the response of different clutches within a category of lake for pre movement using a general linear model, but for overall reaction and spine response to the stimulus the counts were too small to statistically analyze individual clutches. For these I was limited to qualitative visual interpretations of graphical data, looking for broad trends within clutches, or with respect to experimental type, over the four months of trials.

5.3 Results

5.3.1 Simple preference experiments

There is a slight indication that Mayer Lake fish have an affinity for a dark substrate compared to Mayer Pond. In two of five time intervals a significantly greater proportion of Mayer Lake fish are on the dark substrate than Mayer Pond fish (time intervals of 60 and 90 seconds, binomial test $p<0.05$, other intervals $p>0.19$). However, examined over the entire course of the experiment, there is no overall effect due to lake (general linear model with binomial error structure, lake $p=0.8$) or time ($p=0.7$; Figure 41).

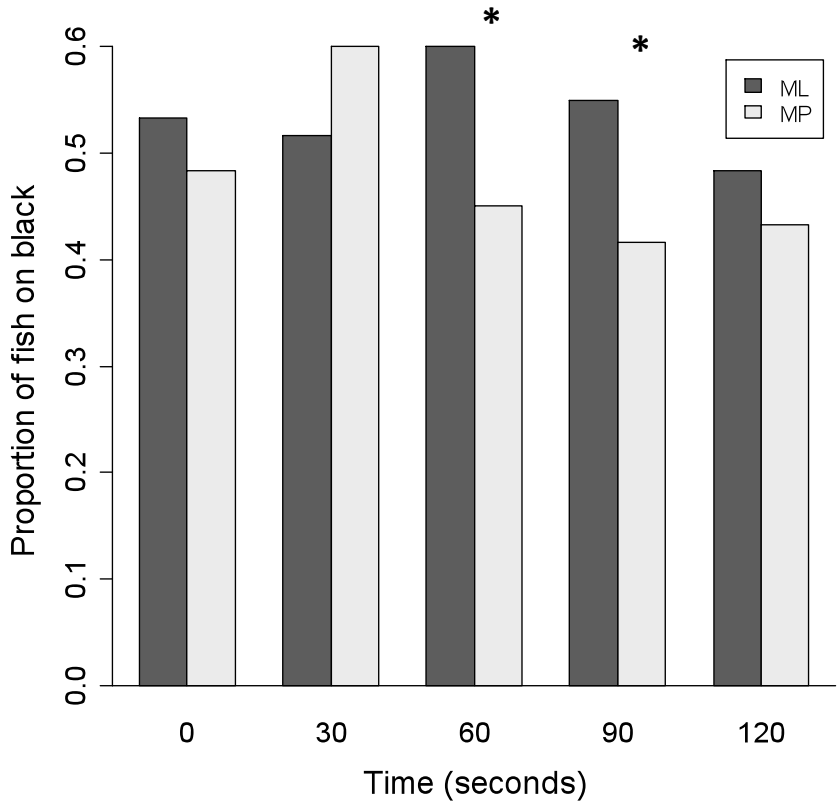


Figure 41: Substrate preference of Mayer Lake (dark bars) and Mayer Pond (light bars). Significant differences (binomial test, $p<0.05$) for each time interval are marked with an *.

Both Mayer Lake and Mayer Pond fish increase in activity over the course of the trials (general linear model, effect due to time $p<0.001$), but Mayer Pond fish are significantly more active than the Mayer Lake population at each time interval (binomial test, all $p<0.001$) and over the entire course of the experiment (general linear model, effect due to lake $p<0.001$;Figure 42).

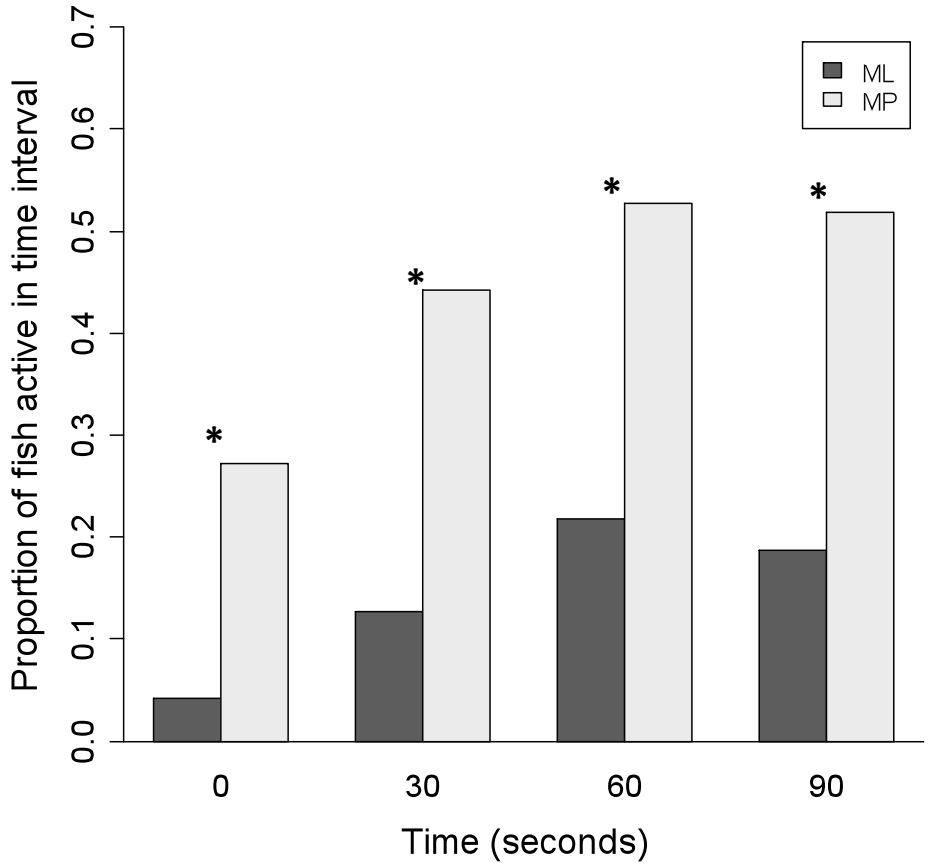


Figure 42: Proportion of Mayer Lake (dark bars) and Mayer Pond (light bars) fish active in substrate preference trials. Significant differences (binomial test, $p<0.05$) for each time interval are marked with an *.

Although there are no differences initially in the cover preference results, Mayer Pond fish are less likely to be under cover by the end of the two minute trials compared to Mayer Lake fish. There are no differences between the proportion of fish under cover for the first three time intervals (binomial test, all p>0.89), nor the fourth 90 second interval (p=0.24), but, at 120 seconds, 52% of Mayer Pond fish are visible, which is significantly more than the 35% of out from under cover Mayer Lake individuals (p=0.013; Figure 43).

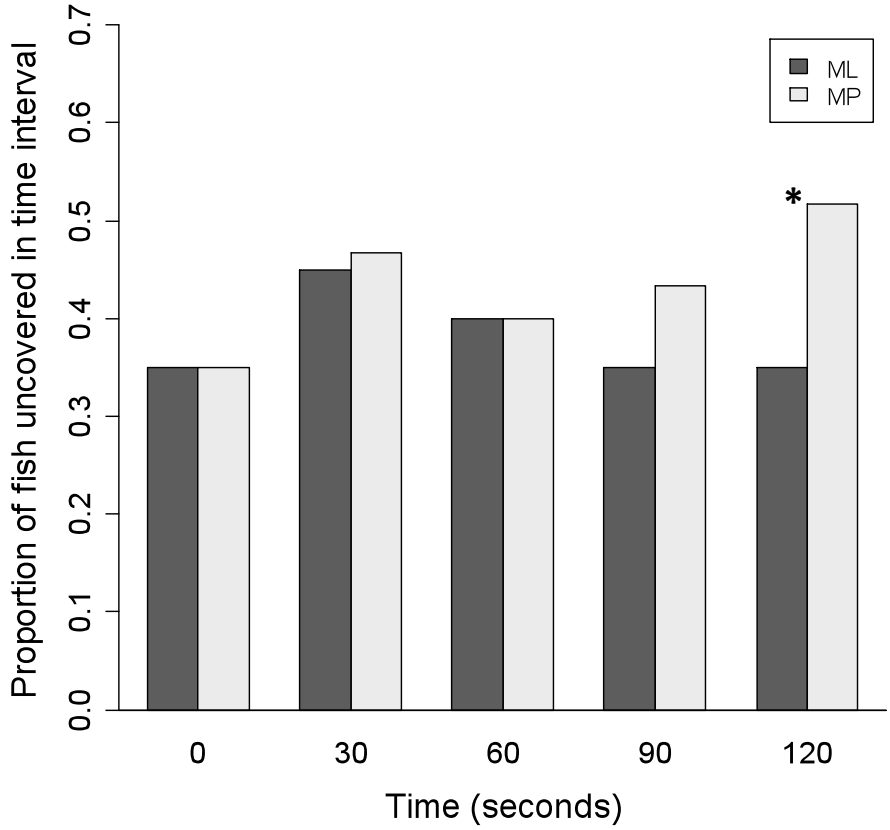


Figure 43: Proportion of Mayer Lake (dark bars) and Mayer Pond (light bars) fish not under cover at 30 second intervals. Significant differences (binomial test, p<0.05) for each time interval are marked with an *

5.3.2 Olfactory cue experiments

Although there are no effects due to the type of stimulus (all main effects and interactions p>0.4), Mayer Lake fish take longer to cross the gate line compared to Mayer Pond individuals. Mayer Pond fish are significantly quicker than Mayer Lake fish in crossing the gate line and exploring the rest of the Y maze (ANOVA, p=0.01; Figure 44). However, this effect is influenced by the number of individuals that did not cross the gate line (and were therefore timed for the maximum five minutes), and this proportion is different between the populations (14% of Mayer Lake individuals versus 4% of Mayer Pond fish). But if these individuals are removed, the effect is still significant (p=0.02).

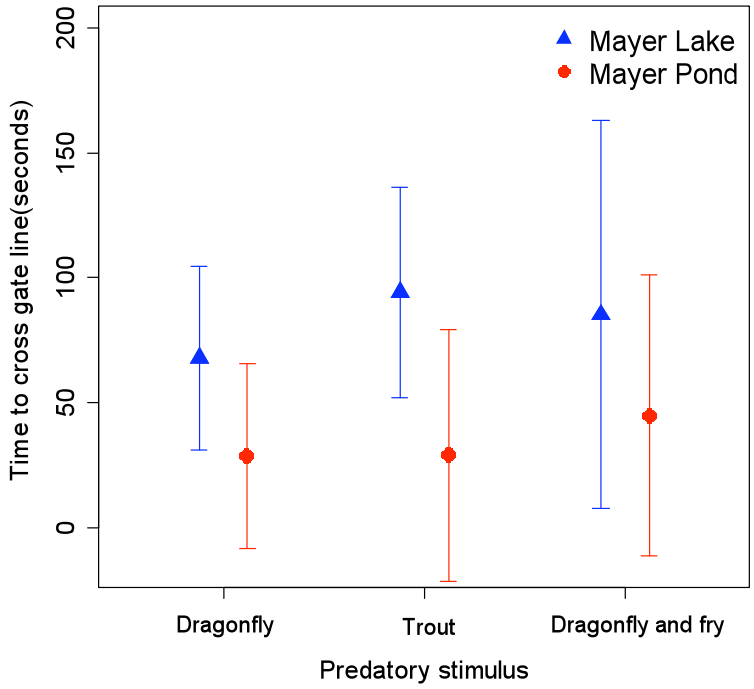


Figure 44: Time (seconds) to cross gateline after the removal of the gate at the beginning of the experiments conducted with three different predatory olfactory cues. Individuals who remained behind the gateline for the entire trial, and were scored 300 seconds by default (four from Mayer Lake and one from Mayer Pond), are excluded from the data presented in this figure.

Mayer Pond individuals are also more active than Mayer Lake during the trials (Figure 45). I defined activity as the events/min, and once again this can be confounded by individuals who did not move across the gateline and were permitted five minutes for a trial. However, regardless of whether these individuals are included or not, Mayer Pond fish are much more active than Mayer Lake individuals ($p<0.01$ with or without individuals who did not cross the gateline). There is no difference with regard to the predatory stimulus (all main effect and interaction effects, $p>0.7$).

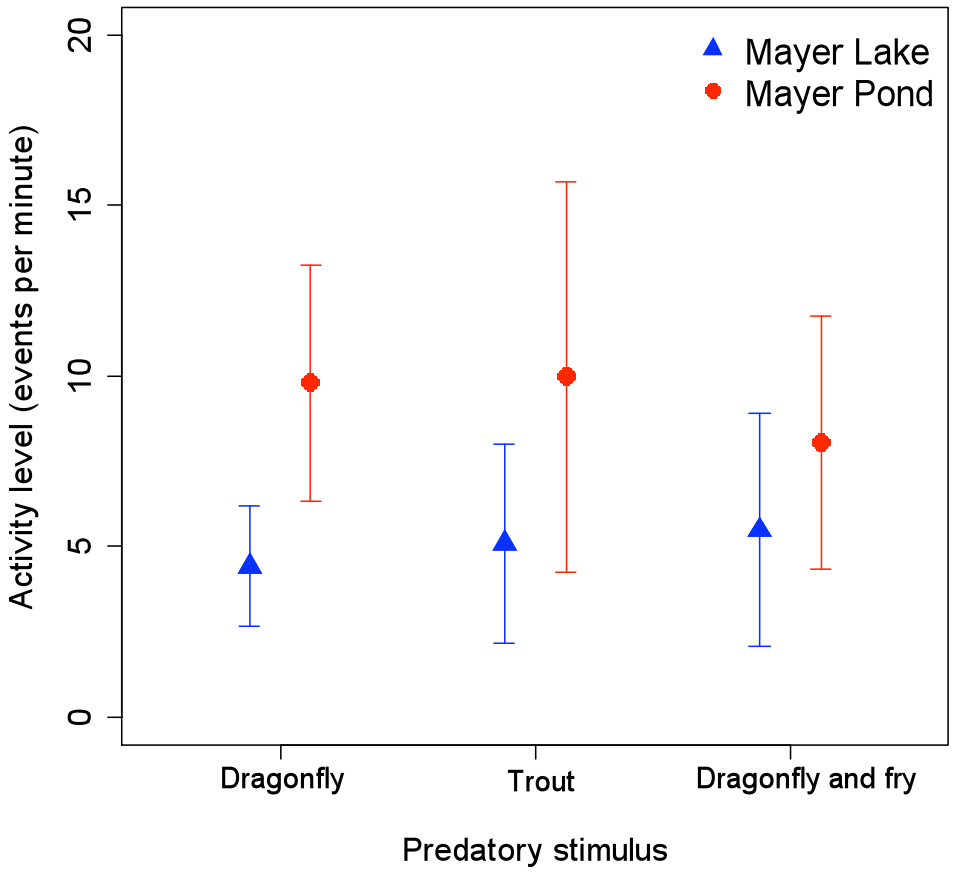


Figure 45: Activity level (events/min – number of times a fish crossed from one section into another, or made a 180 degree change in direction within a section, divided by the length of the trial in minutes) of Mayer Lake fish and Mayer Pond fish during olfactory trials (individuals who did not cross gateline excluded).

There are no differences between the lakes or predatory stimuli with regard to how much time is spent on the opposite side of the maze from the predatory stimulus. Although there is one suggestive instance in which the 95% confidence interval of time spent opposite the predator does not overlap the expected proportion of 0.5 (when Mayer Pond was subjected to the dragonfly larvae; Figure 46), there are no overall significant differences due to lake, predatory stimulus, or any interactions (ANOVA, all $p<0.5$).

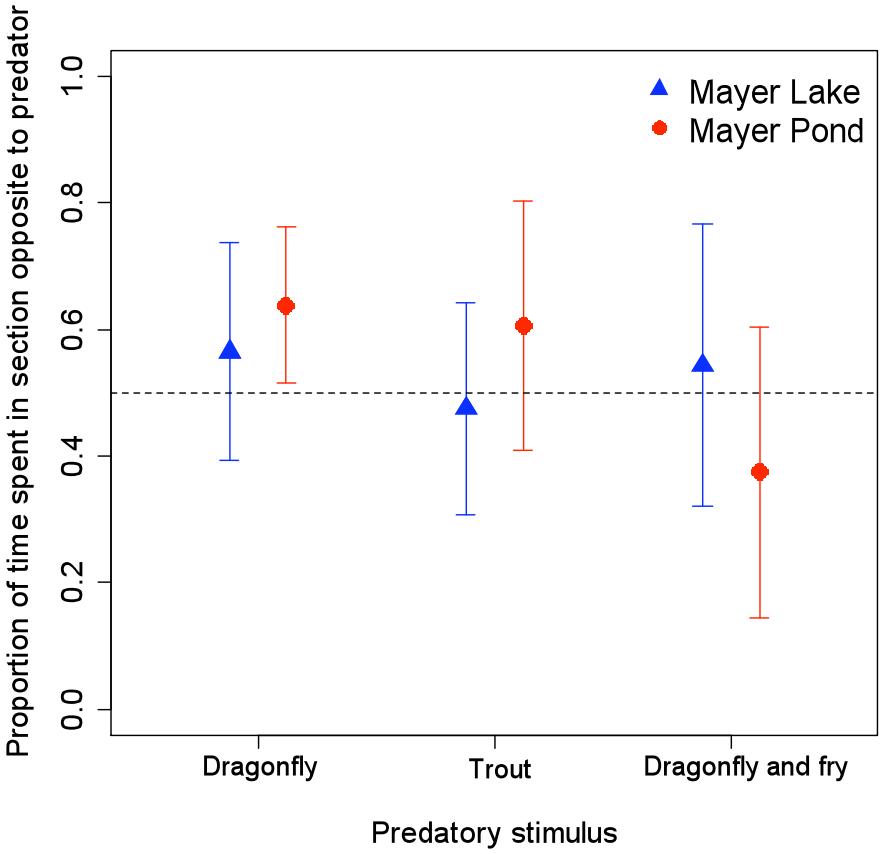


Figure 46: Proportion of trial spent opposite to stimulus with three different potential predatory cues.

5.3.3 Overhead movement, pressure burst and trout water predatory cue experiments

Pre movement

Mayer Pond fish have a higher pre-stimulus activity level than Mayer Lake fish in these predatory stimuli experiments. In all but one of the setups (pressure stimulus, light, clean water) Mayer Pond fish are significantly more active than Mayer Lake specimens (general linear model with Poisson error structure for each experimental type, all $p<0.01$). There are differences in activity between the Mayer Lake clutches, but no consistent trends among clutches (e.g. ML1 always having the highest activity). In many of the setups there is at least one pair-wise comparison where two Mayer Lake clutches cannot be considered equivalent and a few instances where a Mayer Lake clutch is the same as one of the Mayer Pond samples. In one instance (pressure, dark, clean water) MP2b is significantly larger than MP2a. However, despite this variability, in seven of the eight experimental types Mayer Pond fish are more active than Mayer Lake fish. Furthermore, this difference is accentuated in the dark trials. Although it is because Mayer Pond fish becomes more active, not that Mayer Lake fish become less active. This increased difference, like the overall difference between Mayer Lake fish and Mayer Pond fish, seems to be especially driven by the MP2b sample (Figure 47). Another interesting point to note is that although this is before the stimulus, in trials conducted in trout water fish are already being exposed to a predatory cue. However, this does not seem to affect their behavior as activity level in trout water appears equivalent to that in clean water (Figure 47).

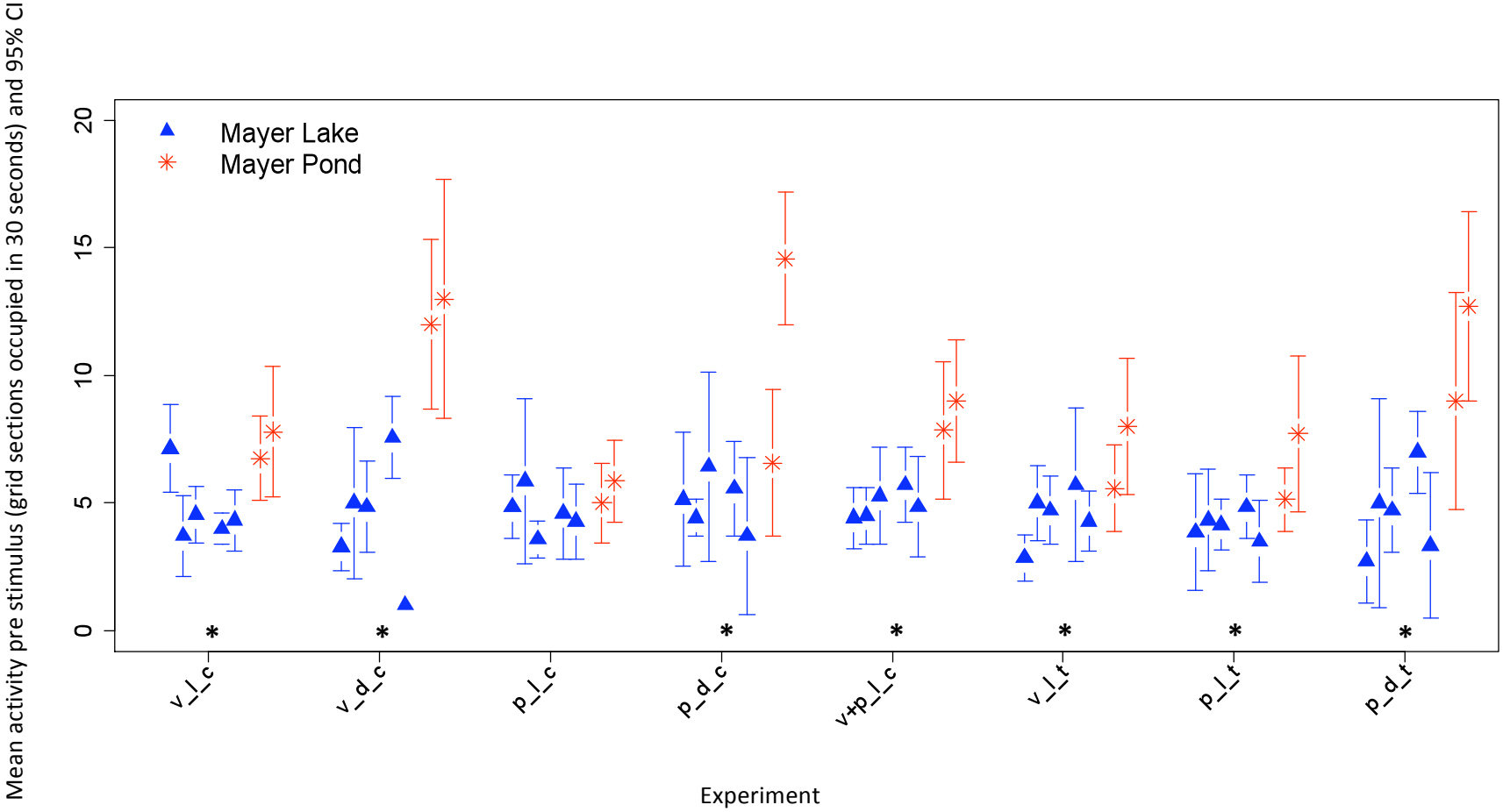
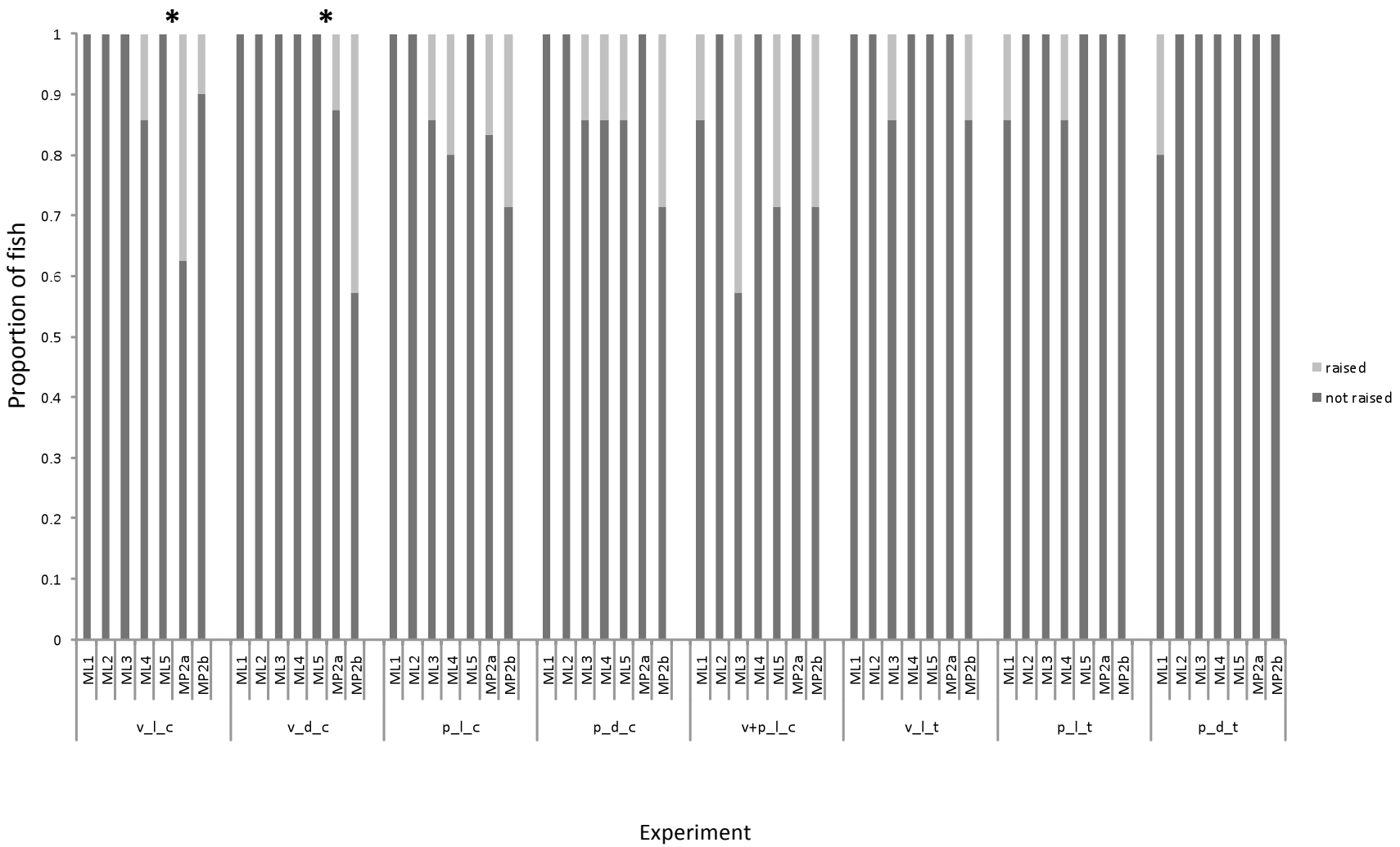


Figure 47: Means and 95% CI of activity level in a 30 second window before the release of the stimulus for Mayer Pond and Mayer Lake fish across eight different experimental setups conducted when the fish were six to 10 months old. The experimental types are represented by: v=visual, p=pressure, or v+p=visual and pressure stimulus, in l=light conditions, or d=dark conditions, with c=clean system water or t=trout water. Significant differences between Mayer Lake and Mayer Pond fish (general linear model with binomial error structure, $p<0.05$) are marked with *.

There is some evidence that Mayer Pond fish are more likely to raise their spines before the stimulus compared to Mayer Lake fish. In the first two trials, Mayer Pond fish are significantly more likely to raise their spines for some part of the 30 second observation window (X^2 test, p=0.03 and p=0.02 for v_l_c and v_d_c trials, respectively). By the third trial Mayer Pond fish are somewhat more likely to raise their spines (X^2 test, p=0.13 for p_l_c trials), but in subsequent trials there are no significant differences (X^2 tests, all p>0.46). In general, fish seem more likely to raise their spines during the visual + pressure, clean water and lit experimental trials, but once again this is before the stimulus has been released, and there should be nothing particularly distinct about this setup, as opposed to trials conducted in the dark or with trout water, and there are no obvious trends relating to those conditions (Figure 48).


Proportion of fish
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
raised
not raised
ML1
ML2
ML3
ML4
ML5
MP2a
MP2b
v_l_c
v_d_c
p_l_c
p_d_c
v+p_l_c
v_l_t
p_l_t
p_d_t
Experiment

For the most part, there are no differences between the response of Mayer Lake and Mayer Pond to the various predatory stimuli. There is one experiment (pressure, light, clean water) where Mayer Pond is less likely to show an escape behaviour (77% of fish show no response, while only 15% display an escape burst) than Mayer Lake (36% of fish show an escape burst, and an additional 27% freeze) and this difference is significant (X^2_2=6.2, p=0.045). However, in the rest of the trials there is no difference between the two populations (X^2 test tests, all p>0.18; Figure 49).

Examined across all clutches, the strongest antipredator response (particularly the number of 'escape bursts') occurs in the visual, light, trout water trials and, secondarily, in the visual+pressure, light, clean water experiments (Figure 49). These are trials where a visual predatory stimulus was paired with another cue, either trout olfactory cues, or pressure cues. As the next experimental type (pressure, light, trout) was carried out the next day (Table 10) and did not show similar trends, this result is unlikely the result of some external environmental cue or stress during this time period. Note also that there is almost no response to the visual stimulus trials conducted in the dark, which were intended to serve as a control testing that the environment was indeed too dark for visual cues, and that the overhead stimulus didn't provide other, non-visual, stimuli. A few fish were scored as 'stopping', but this probably reflects the background level of this behaviour, rather than an actual stimulus response (an important consideration when evaluating 'stopping' in other trials; Figure 49).

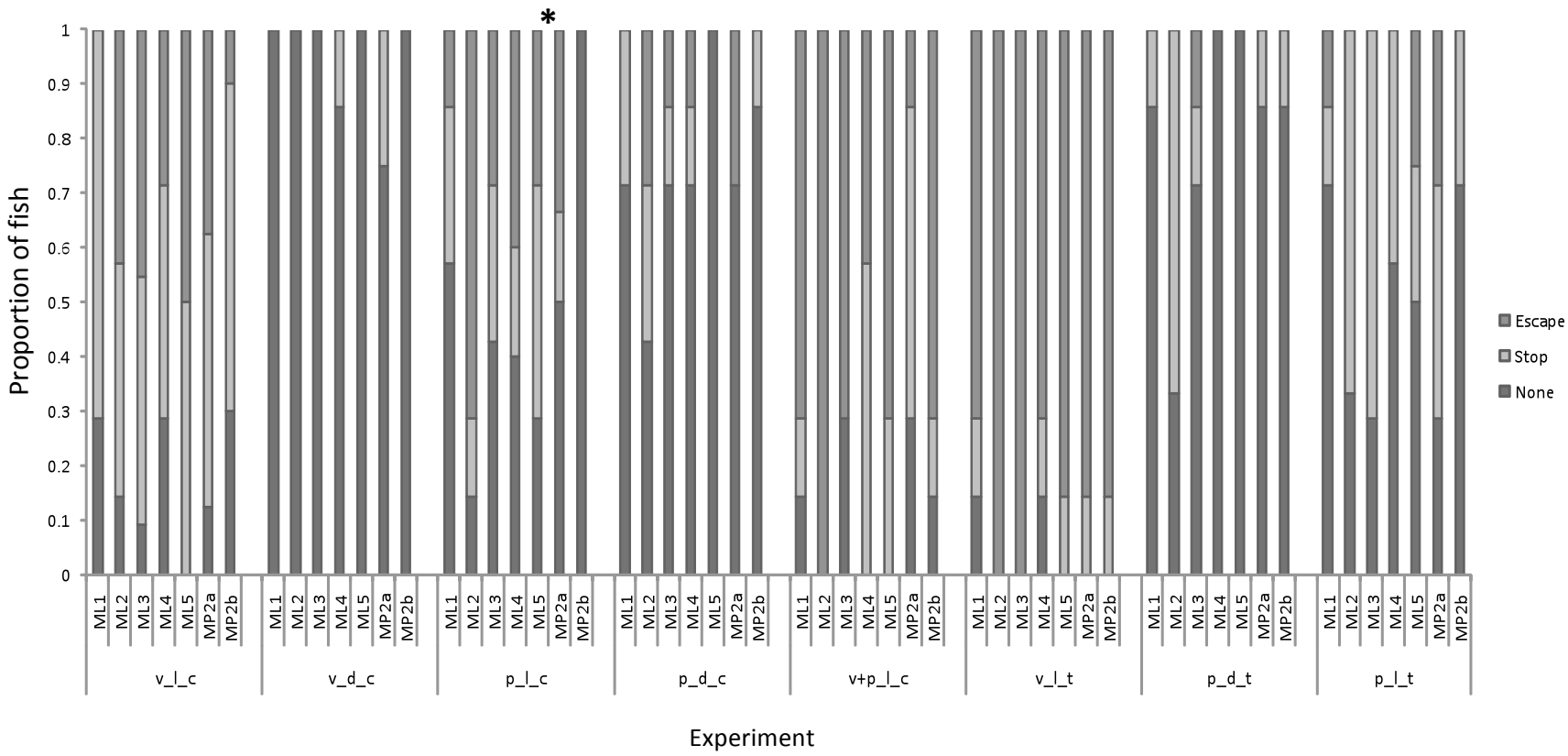


Figure 49: Response (Escape = burst of swimming; Stop = freezing or slowly sinking away from the stimulus; None= no adverse reaction) to different predatory stimuli of fish from Mayer Pond and Mayer Lake across eight different experimental setups conducted when the fish were six to 10 months old. The experimental types are represented by: v=visual, p=pressure, or v+p=visual and pressure stimulus, in l=light conditions, or d=dark conditions, with c=clean system water, or t=trout water. Significant differences (X^2 test, $p<0.05$) between populations are marked with an *.

There is no overall difference between Mayer Lake and Mayer Pond in their spine response to the different predatory stimuli (X^2 test, all $p>0.11$). However, there are some interesting results if we look at the trends across different experimental types (Figure 50).

The trends in spine response reflect those seen in the overall response to predatory stimuli displayed above. There are very few individuals who raise their spines for the visual stimulus in the dark indicating that differences in other setups are not just the result of chance. And, in general, there is a moderately high rate of spine response to the pressure stimulus, and the highest response to the overhead visual stimulus. Furthermore, the pairing of two stimuli (either visual and pressure, or each with trout water) also slightly increases the likelihood of this antipredator behaviour, but the difference is not as distinctive as in the overall escape response (Figure 50).

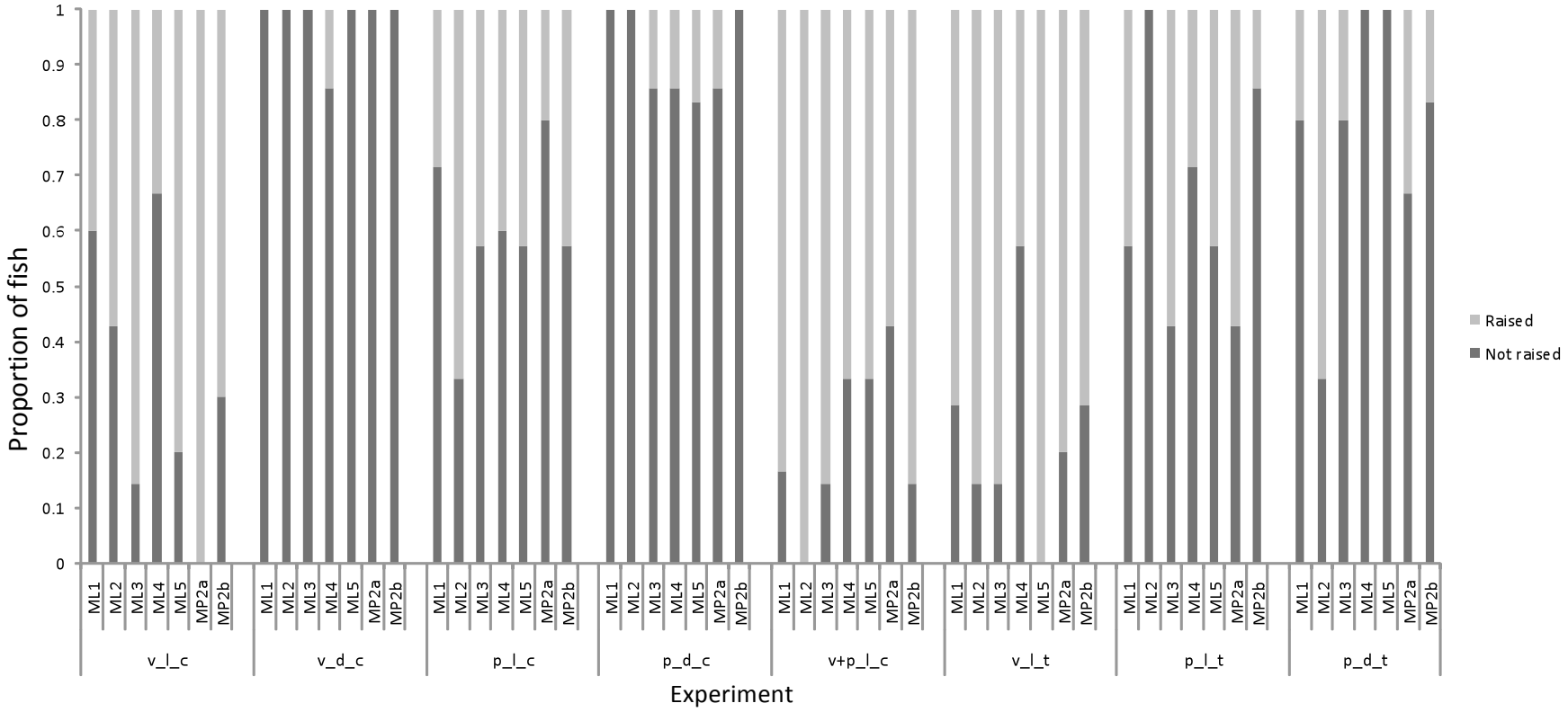


Figure 50: Breakdown of spine raising between clutches in response to different predatory stimuli over four months of trials. The experimental types are represented by: v=visual, p=pressure, or v+p=visual and pressure stimulus, in l=light conditions, or d=dark conditions, with c=clean system water, or t=trout water.

Mayer Lake fish generally take longer to recover following a stressful stimulus than Mayer Pond individuals. Although this difference is only close to significant in two situations (T test comparing Mayer Lake and Mayer Pond fish for recovery time in a given trial, v_l_c and p_l_c, p=0.07; v_l_t, p=0.09), when examined across all trials the Mayer Pond samples consistently have some of the shortest recovery times of the seven lab-raised groups (Figure 51). This replicated trend over time and experimental setup supports the inference that Mayer Lake fish generally take longer to recover than Mayer Pond individuals following a stressful stimulus.

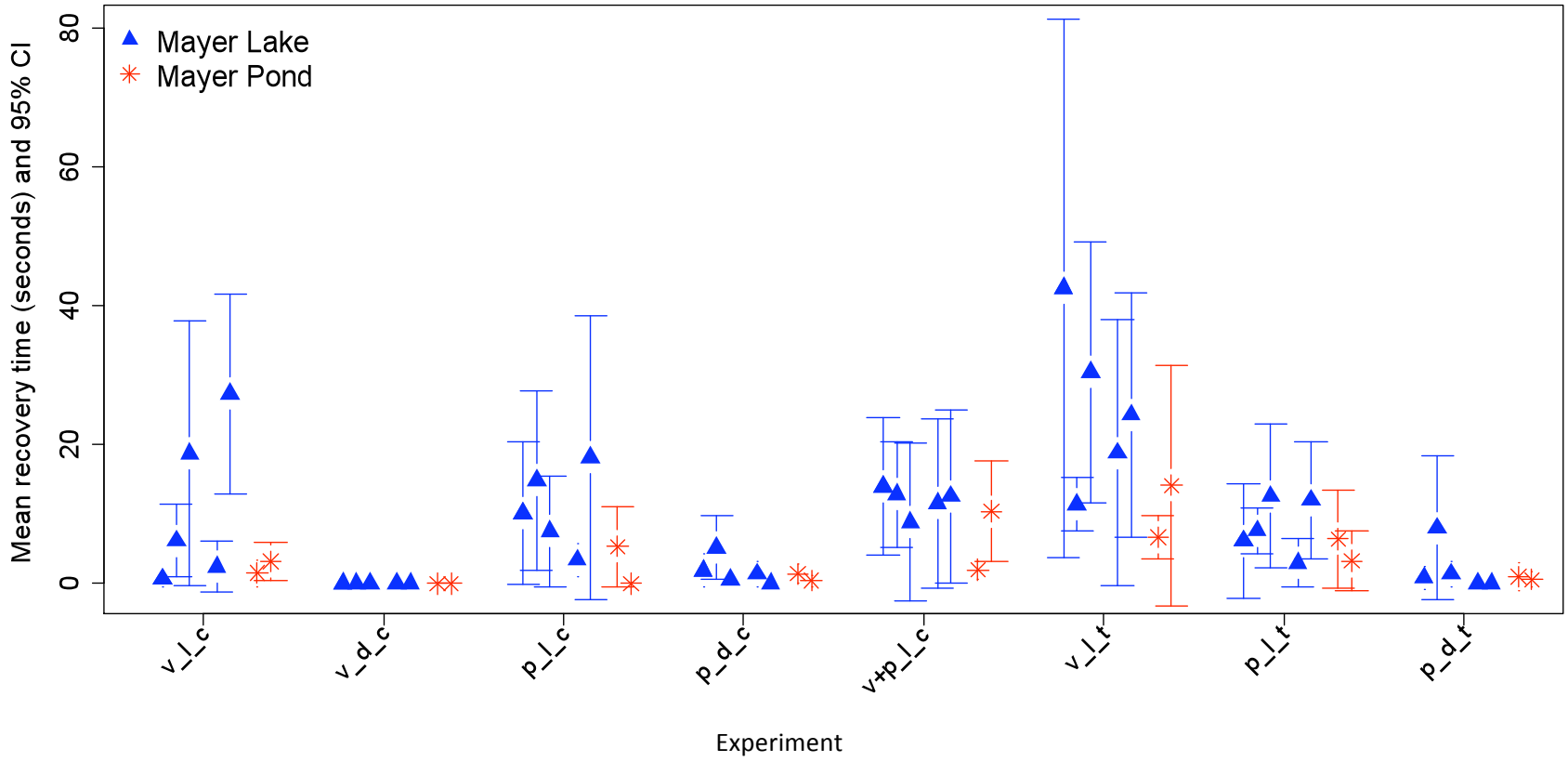


Figure 51: Means and 95% CI of time to recover normal behaviour following the stimulus for Mayer Pond and Mayer Lake across eight different experimental setups conducted when the fish were six to 10 months old. The experimental types are represented by: v=visual, p=pressure, or v+p=visual and pressure stimulus, in l=light conditions, or d=dark conditions, with c=clean system water, or t=trout water.

5.4 Discussion

Mayer Lake is a dark water habitat dominated by vertebrate predators such as cutthroat trout and diving birds like common loons, while Mayer Pond is a turbid water environment with invertebrate predators instead. The different selective pressures exerted in these two habitats led me to predict a variety of behavioural differences between Mayer Lake and Mayer Pond fish. Specifically, I expected Mayer Lake fish to demonstrate a preference for dark habitats (greater preference for dark substrates, more active in dark conditions), be more likely to hide under cover due to the increased risk of overhead predation, and to show greater antipredator response to trout olfactory cues and overhead movement mimicking the attack of a diving bird. Mayer Pond fish, historically lacking these predators, were instead expected to show an increased response only to invertebrate olfactory cues.

Only two of these predictions, the darker substrate and the increased cover preference of Mayer Lake compared to Mayer Pond, were somewhat supported. In two of the five observation intervals in the substrate preference experiment Mayer Lake fish were significantly more likely to be found on dark substrate compared to Mayer Pond fish. And by the end of the cover preference experiments, Mayer Pond fish were significantly more likely to have come out of cover compared to Mayer Lake fish. However, across all of the experiments with different predatory stimuli including olfactory cues, visual cues, and overhead cues, I found no specific differences between the populations. There was one consistent and repeated difference between Mayer Lake and Mayer Pond, although it wasn't one I originally expected, with respect to activity level.

The data reflecting the increased activity of Mayer Pond in novel environments are the most consistent indications of behavioural differences between Mayer Lake and Mayer Pond. From the first substrate preference experiments when the fry were only one month old, through the olfactory Y maze chamber experiments at two months, and even to a degree throughout the various predatory stimulus trials carried out when the fish were from six to 10 months old, there is a consistent and replicated trend that fish from Mayer Pond are more active than fish from Mayer Lake. A greater activity level in fish from a lower predator environment has been previously observed and some attribute this to be a greater willingness to explore novel environments (Brown and Braithwaite 2004), while to others it is simply being more 'hasty' by

taking less time to make potentially risky decisions (Burns and Rodd 2008). Regardless of underlying motivation, the main consistent behavioural difference between Mayer Lake and Mayer Pond is in their overall activity level.

However, closely related to activity level is a measure of 'recovery time'. The time to recover following a stressful stimulus is often lower in populations with less predation pressure (Giles 1983a, Tulley and Huntingford 1986). There is some indication that Mayer Pond recovers more quickly than Mayer Lake when it was explicitly measured following the predatory stimulus trials, and I would argue that all of my experimental measures of activity level, even before the specific experimental stimulus, are also an indication of recovery time following the unquestionably stressful experience of being transferred to the experimental chamber. This inference is supported by the earliest preference experiments where activity level continued to rise over the course of experiment, presumably as fish relaxed following the stressful transfer and acclimatized to their new environments. Therefore, there is additional evidence that lab-raised Mayer Pond fish recover more quickly than Mayer Lake fish following a stressful event, as well their overall activity level within a novel environment.

A quicker recovery and greater activity in a novel environment has been documented in other low predation populations (Kennedy *et al.* 1994, Brown and Braithwaite 2004), and makes sense in light of predator sensitive foraging theory (Miller 2002). Much work has been done on optimal foraging theory, and how predation risk affects foraging choices (Giles 1987, Gilliam and Fraser 1987, Gotceitas and Godin 1991, Bishop and Brown 1992). Simply put, if the risk of predation is lower, an individual will resume foraging more quickly, both due to possible competition, and simply to increase overall energy gain (Godin and Sproul 1988). While the relation between activity and predation risk has been documented before, this study is valuable in that it used lab-raised fish indicating these differences may have heritable components, in addition to the interesting and valuable colonization history and ecological understanding of the Mayer Lake and Mayer Pond environment.

Additionally, what can provide further insight are the general trends across all the different clutches with regards to their response to different predatory stimuli. Both populations show a strong adverse response, measured both by their escape response and their spine response - an accurate measure of perceived predatory threat (Giles and Huntingford 1984, Huntingford and Giles 1987) - to an overhead moving object.

Antipredator behaviour in response to overhead movement is well documented. The escape from an overhead shadow is stereotyped as the 'Mauthner response' in fish in general, and in stickleback experimenters have found a consistent response to everything from a dipping heron's bill (Giles and Huntingford 1984, Alvarez and Bell 2007) to a silhouette of a predatory bird (Tulley and Huntingford 1986, Messler *et al.* 2007). It is interesting that the shape used in my trials, which has no specific resemblance to a predatory form, still elicits a response. Furthermore, the response to this visual cue seems to be amplified by the addition of olfactory or pressure cues.

Olfactory predatory cues are important for the assessment of predation risk (Ylonen *et al.* 2007). Although my earliest olfactory experiments did not show conclusive results, this may be a result of methodological issues. Water likely mixed when exiting the Y arms preventing me from identifying 'time spent opposite to predator' and olfactory cues, especially skin swabs taken from the trout, may simply have not been strong enough. Regardless, the addition of trout water to the visual stimulus seemed to amplify the response in the later predatory cue experiments.

Similarly, the addition of pressure to the visual stimulus amplified the antipredator response. Fish are relatively unique in possessing a lateral line system that allows accurate detection of subtle differences in pressure (Kotrschal *et al.* 1998). This system plays an important role in predator detection and evasion (McHenry *et al.* 2009), and its antipredator role supported by my data with the relatively high escape response to the pressure predatory cues. When paired with a visual stimulus I observed an even stronger response.

Responding to a predator is energetically costly (Helfman 1989, Brown *et al.* 2005, Ajie *et al.* 2007), although not responding when necessary has obviously even greater costs (Abrahams and Dill 1989). It is both reasonable and interesting that I saw a 'graded' response to predatory stimuli, with additional stimuli provoking a greater response. Presumably, additional indications of an imminent attack, be it olfactory or pressure cues, increases the likelihood of an extreme response such as an energetically costly escape response. Interestingly, there is less of a difference in the other measure of perceived predatory threat – spine response – with additional predatory cues. The level of spine raising is quite low before the stimulus, and many different predatory cues elicit a spine raising response. However, as this antipredator measure is not very energetically costly, but very effective (Hoogland *et al.* 1956), it makes sense that the fish would take this precaution regardless of attack probability. It is only when there are greater

energetic consequences that additional cues make a considerable difference in the likelihood of eliciting a stronger antipredator response such as quick escape bursts.

A weakness in this study is that the Mayer Pond population is represented by only one clutch. While the original size of the clutch (~330 eggs) is sufficiently large that it may represent eggs from more than one female, it is still within the potential clutch size range of the larger sticklebacks (Baker *et al.* 2008). Additional lab-raised individuals from Mayer Pond are needed to determine if the behaviour demonstrated by the fish I studied is representative of the population in general.

Although I did not find the predator-specific differences that I expected, there is evidence from these lab-raised individuals that heritable differences in activity level, willingness to explore a novel environment, and recovery time following a stressful stimulus have developed in Mayer Pond fish within 14 years after Mayer Lake individuals were transplanted into a fundamentally different habitat. Although these lab-raised samples are small, and I only had one clutch representing Mayer Pond, if these results are substantiated with further trials these differences, although subtle, are a very interesting indication of rapid evolutionary change in behaviour. Further tests may not only support or refute my findings but, as they will occur even longer after the original transplant, perhaps these differences (or others such as predator specific responses) will be even more noticeable.

Chapter 6: Integration and Synthesis

Throughout the history of life on Earth there have been countless colonization events as species exploit novel opportunities in an ever-changing world. Some events, such as the recession of glaciers following the end of the last ice age approximately 11,000 years ago, provide a relatively recent and geographically widespread opportunity for the colonization of previously unavailable habitat. The threespine stickleback is one species that exploited these opportunities on a massive scale, colonizing streams, rivers, and lakes around the northern hemisphere on a journey from marine to the newly available freshwater habitats. Knowing the time since colonization (approximately 11,000 years), the ancestral form (fully plated marine stickleback), and geographically widespread correlations between the habitat characteristics and resulting phenotype of stickleback in a number of independent, replicated, natural experiments have been of tremendous use for studies of ecology and evolution. However, still remaining are questions such as: at what rate do these changes take place? Were phenotypic modifications complete after 10 years? Or are even perhaps still ongoing 11,000 years later? Tied in with those are questions about mechanism: did the phenotype change by slow, incremental, modifications of the genetic structure in the population caused by differential fitness and natural selection? Or are features phenotypically plastic and changed immediately in response to new environmental conditions?

Seventeen years ago, T.E. Reimchen transplanted 100 sticklebacks from Mayer Lake to Mayer Pond in order to investigate the consequences of a drastic alteration in selective landscape on the phenotype of this well studied species, and to gain insight into the rates and mechanisms of evolutionary change. Fourteen years later, I began working on identifying the presence of any morphological and behavioural changes in Mayer Pond fish compared to Mayer Lake individuals, as well as the mechanisms by which changes occurred over such a short time frame. Given the previous associations between habitat type and resulting morphological and behavioural phenotype in threespine stickleback, I began with a number of predictions as to the direction of changes in Mayer Pond individuals.

In Mayer Lake, the predation regime is primarily comprised of fish predators such as cutthroat trout and diving bird predators such as the common loon, while in Mayer Pond macroinvertebrate predators are a primary source of mortality. Morphologically, I expected a

reduction of bony armoured structures (size of ascending process, pelvic plate, and size and presence of lateral plates) as well as spine length, due to selective pressures imposed by grasping invertebrate predators and the costly maintenance of potentially unnecessary structures. Behaviourally, I expected fish from Mayer Pond would be more likely to respond to invertebrate, compared to trout, olfactory cues, and less likely to respond to overhead movement and possibly pressure burst predatory stimuli.

Mayer Pond is also different from Mayer Lake in prey field and habitat type. Mayer Pond more closely resembles a benthic habitat, with extensive vegetation and the presence of benthic type prey, while Mayer Pond stickleback primarily live in a limnetic environment and eat planktonic prey. Morphological specialization in response to limnetic and benthic environments has been well studied and I therefore expected Mayer Pond individuals to have more benthic features, such as larger eyes, shorter jaws, and shorter and fewer gill rakers. Behaviourally, I thought that Mayer Lake being a dark water environment, and Mayer Pond being a turbid water habitat, might result in differences in substrate preference (with Mayer Lake preferring darker substrates).

Many of the morphological predictions were accurate. In defensive structures I found evidence for a reduction in lateral plate presence, and to some degree lateral plate size, and also a reduction in the size of the ascending process area and the length of the dorsal and pelvic spines, as well as the width of the first dorsal spine. However, I found no evidence of change in the pelvic plate area or length. In feeding structures, I found indications that Mayer Pond fish have larger eyes, and shorter gill rakers, but, contrary to expectations, no change in jaw length or gill raker number. In many of features however, Mayer Pond fish have morphologically diverged from the Mayer Lake baseline in the expected direction with reduced defensive features and more benthic type feeding structures.

There are behavioral differences between Mayer Pond and Mayer Lake fish, but not as many as I expected. The most recurrent, and therefore substantive, difference between the two populations was a difference in recovery time and activity in an unfamiliar environment. From one to 10 months of age and across many experimental setups, Mayer Pond fish were significantly quicker to recover following a stressful stimulus and more active in a novel environment. While Mayer Lake fish displayed a preference for dark substrates compared to Mayer Pond individuals, there were no detectable differences in any other stimuli specific cues such as different predator olfactory cues or mechanisms of attack including overhead or

pressure stimuli. Perhaps these predatory specific responses are developed primarily through experience, and would not be expected to be detectable in lab-raised fish, especially after such a short period of separation. The detectable increase in activity level in lab-raised Mayer Pond fish therefore implies that there is a tremendous gain in fitness from taking advantage of additional opportunities to forage when predation is low, so much so that there are heritable differences influencing this behavioural characteristic within 14 years since transplantation.

In addition to investigating any morphological and behavioural differences between individuals from Mayer Lake and Mayer Pond, I was also interested in the mechanism (rapid evolutionary change by natural selection vs. phenotypic plasticity) responsible for any changes. As fish were raised under the same conditions for the behavioural experiments, I concluded any differences were the result of heritable components for that aspect of behaviour that resulted from rapid evolutionary change after only 14 years of separation between the two populations. As behaviour is quite plastic (for review see Foster 1999), and Mayer Lake and Mayer Pond have been separated for such a short time, it is perhaps understandable that I did not detect more differences between the two groups when raised in the lab. Other studies examining the effect of predation pressure type and intensity on behaviour that have used similar predatory stimuli and behavioural measures, but have detected more substantive differences, have used fish with some native environmental experience, which has inherent problems discussed earlier, or have examined populations that have been separated for much longer (For example see Huntingford 1982, Tulley and Huntingford 1986). Also, my comparisons were perhaps limited by having only one clutch from Mayer Pond. With greater time after transplant and an increased sample size, as well as including comparisons of wild-caught individuals, the mechanisms of behavioural changes in Mayer Pond can be more thoroughly investigated.

Most of the morphological features, with the exception of lateral plate presence, in which I identified changes in Mayer Pond fish occurred immediately in the first generation and persisted relatively unchanged in subsequent samples, especially those that showed the strongest change such as gill raker and spine length. The addition of lab-raised fish provides more insight as to whether these immediate changes are the result of intense selection on, or restricted genetic variability within, the founding population, or if they are phenotypically plastic responses to the new environment.

There is almost no indication that any of the morphological differences observed in wild-caught fish persist when reared under identical conditions. If differences were the result of intense selection on the colonizing individuals, or possibly a founder's effect caused by limited genetic variability in the founding 100 fish, then we would expect differences to persist in the lab-raised individuals. That they do not lends support the inference that morphological changes in Mayer Pond are primarily the result of phenotypic plasticity.

There is a growing interest in phenotypic plasticity and increasing recognition of plasticity as an important concept in evolutionary biology (Pigliucci 2005). Although the importance of plasticity as an adaptive strategy to deal with environmental variation is increasing, studies of natural systems lag behind theory (Day and McPhail 1996). There are also few studies of plasticity in stickleback, with two artificially modifying environmental cues in the lab (Day *et al.* 1994, Day and McPhail 1996), and one examining plasticity in a recently colonizing population (Baker and Foster 2002). My work is a useful addition as it combines samples from natural populations, including one of the most derived populations in the species (Moodie 1972), with lab-raised comparisons, as well as knowledge of the ecological conditions of the two habitats and details of the colonization.

It is during colonization events in which phenotypic plasticity may be most important (Price *et al.* 2003). If we consider the adaptive landscape experienced by a population, the extreme shifts in the adaptive peak experienced when colonizing new habitats can result in extinction unless populations have extreme individuals able to survive in the new conditions, or individuals that are phenotypically plastic and can modify their phenotype in response to the new environment (Price *et al.* 2003). The persistence and success of stickleback in Mayer Pond after such abrupt alterations in their selective landscape would have been aided by phenotypic plasticity.

Of the apparently phenotypically plastic features in my work – spine and gill raker length, eye dimensions, and ascending process area – only one, gill raker length, has been explicitly investigated for plasticity (Day *et al.* 1994, Day and McPhail 1996). Day *et al.* (1994) found that by altering the diet of lab-raised benthic and limnetic stickleback the feeding morphology, including gill raker length, would begin to resemble the alternative form (e.g. limnetics fed benthic food would have more benthic like structures than if fed limnetic food). However, a follow up investigation by Day and McPhail (1996) using adult wild-caught adult fish found no difference in most feeding structures including gill raker length. From this they

concluded that these structures likely represent non-labile plasticity (i.e. the feature can be altered by environmental input, but only for a limited time during development, as opposed to labile plasticity where a feature is always responsive to environmental changes). This is similar to findings by Meyer (1987), who found phenotypically plastic feeding morphology in cichlids, but only up to a certain point in development. Furthermore, Day and McPhail (1996) felt that diet induced morphologically changes probably result from plastic responses of bone or muscle to novel mechanical stress imposed during critical periods of development during ontogeny. The altered mechanics and stress of eating different prey in Mayer Pond may be the proximate cause inducing a different gill raker length.

Although phenotypic plasticity is defined as one genotype producing a range of phenotypes depending on environmental conditions, it is incorrect to separate environmentally induced plasticity from genetics, and to view the two as opposing forces in evolutionary change as in the classic 'nature vs. nurture' debate (Pigliucci 2005). Plasticity has a genetic basis and likely evolves as an adaptive response to variable environmental conditions (Price *et al.* 2003). Furthermore, moderate amounts of plasticity can encourage further genetic change by natural selection. Without plasticity, individuals might not be able to survive abrupt alterations in selective landscape, but, having survived the initial change, moderate amounts of plasticity may not be sufficient to result in the optimal phenotype for a particular environment's adaptive peak (Price *et al.* 2003). In these situations, termed genetic assimilation, further genetic changes in the population allow individuals to reach this optimal fitness peak and these genetic changes eventually predominate in the population (Price *et al.* 2003) as potentially costly plasticity is lost (Pigliucci 2005). Plasticity may thereby represent the first step in evolution by natural selection (West-Eberhard 2005). The rapid morphological changes in Mayer Pond may be the result of plasticity now, but perhaps in time will become more genetically fixed in this population. This may also help explain why many previous studies have found traits such as gill raker and spine length to have high heritable components, as opposed to my findings of extensive plasticity, since they compared much more established populations (Hagen 1973, Hatfield 1997, Aguirre *et al.* 2004).

I did not predict that Mayer Lake would have this high potential for plasticity. The potential for plasticity is primarily related to environmental variability in a habitat (Price *et al.* 2003) and, as plasticity is often costly (for reviews see Pigliucci 2005), I did not have expect plasticity to be maintained in a population which, given the morphological equivalence of fish

taken from 1929 and 2003, appears to be quite constant (also see morphological equivalence of samples in Moodie 1972). Furthermore, Day and McPhail (1996) suggest that differences in plasticity potential may be related to lifespan, with shorter lived individuals experiencing greater inter-generational variability and therefore maintaining a higher potential for plasticity to deal with unpredictable environments. Mayer Lake not only appears to be a relatively constant environment, but resident sticklebacks are among some of the longest lived of all stickleback, not reproducing until the third year (Moodie 1984) - much later than the typical stickleback (Baker *et al.* 2008). Given the longevity and apparent environmental stability of Mayer Lake, the morphological plasticity exhibited when these fish were reared in Mayer Pond, and the reversion of any changes when reared in common conditions in the lab, is surprising. Perhaps plasticity is not that costly to maintain, or perhaps, as Mayer Lake is so large, there is sufficient spatial variability across the lake and migration between these areas that plasticity has been maintained. Regardless, there is a tremendous potential for phenotypic plasticity in Mayer Lake fish, and perhaps the rest of the threespine stickleback species as well.

Complementing my investigations of morphology and behaviour, which infer evolutionary mechanisms by examining rates of change as well as comparing wild-caught and lab-raised individuals, is current genetic work being carried out on these populations. Initial genotype data (~1200 genome-wide single nucleotide polymorphisms) indicate two important points. First, there is almost no change in overall heterozygosity between Mayer Lake and Mayer Pond samples, and the overall level of genetic differentiation between the Lake and Pond is small (overall Fst = 0.031; B. Deagle, F. Jones, D. Kingsley, T. Reimchen, unpublished data). These data indicate that the founder effect was not a major driver of observed phenotypic changes, and supports my inferences that the immediate changes are more likely the result of phenotypic plasticity (B.Deagle, personal communication). Secondly, some notable changes in allele frequencies at specific loci have occurred between the Mayer Pond samples collected in 1998 and 2007 (B.Deagle, personal communication). This indicates the potential for heritable changes caused by divergent selection in the two habitats, although at this point the morphological or behavioural associations with these loci are unknown.

Following the recession of the glacial ice sheets at the end of the Pleistocene, threespine stickleback successfully colonized a wide diversity of habitats and underwent massive adaptive radiation (review in Wootton 2009). I found evidence for rapid evolutionary change including the immediate and progressive decline of the presence of the second lateral plate, as well as

presumably heritable behavioural differences that occurred in a population of threespine stickleback after only 14 years since colonizing a fundamentally different environment. I also found extensive evidence from wild-caught and lab-raised specimens for phenotypically plastic morphologically changes. Both rapid evolutionary change and phenotypic plasticity have allowed Mayer Pond inhabitants to not only survive, but to thrive (as evidenced by an increase from 100 founding individuals to a population size of several thousand over 15 years). This system may therefore exemplify the potential for phenotypic flexibility and rapid evolutionary change present in threespine stickleback that has likely resulted in the diversity and success of this species in so many other colonization situations.

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