

Evolutionary history of threespine sticklebacks (*Gasterosteus* spp.) in British Columbia: insights from a physiological clock

Rees Kassen, Dolph Schluter, and John Donald McPhail

Abstract: Geologic and allozyme evidence suggests that threespine sticklebacks (*Gasterosteus aculeatus* complex) in low-lying southwestern British Columbia lakes were founded during two incursions of marine sticklebacks after the retreat of the Pleistocene glaciers (the double-invasion hypothesis). We used the salinity tolerance of embryos, measured as hatchability in salt water, to establish the relative order of freshwater invasion by marine sticklebacks and to test the double-invasion hypothesis. Limnetics and an anadromous population hatched nearly equivalent numbers of young in salt water as in fresh water, whereas benthics and one solitary freshwater population had low hatchability in salt water. We also found that eggs from freshwater populations were larger than those from marine populations and limnetics had smaller eggs than benthics and the solitary population. These results support the double-invasion hypothesis and suggest a trend of increasing egg size with increasing time spent in fresh water.

Résumé : Des données géologiques et des données sur les allozymes indiquent que les Épinoches à trois épines (complexe *Gasterosteus aculeatus*) des lacs de basse altitude du sud-ouest de la Colombie-Britannique sont apparues à la suite d'incursions en eau douce d'épinoches marines après le retrait des glaciations du Pléistocène (hypothèse de la double invasion). Nous avons estimé la tolérance des embryons à la salinité en évaluant leur potentiel d'éclosion en eau de mer, dans le but d'établir l'ordre relatif des arrivées en eau douce d'épinoches marines et d'éprouver l'hypothèse de la double invasion. Les épinoches limnétiques et une population anadrome ont donné lieu à des nombres équivalents de poissons néonates en eau de mer et en eau douce, alors que les épinoches benthiques et une population dulcicole isolée ont manifesté un faible taux d'éclosion en eau de mer. Nous avons également constaté que les oeufs des populations dulcicoles étaient plus gros que ceux des populations marines et que les épinoches limnétiques ont donné des oeufs plus petits que les poissons benthiques et que la population dulcicole isolée. Ces résultats appuient l'hypothèse de la double invasion et indiquent la possibilité d'une corrélation entre la taille des oeufs et la durée du séjour en eau douce.

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Introduction

Postglacial invasions of fresh water by marine fish in North America provide ample evidence of adaptive radiations resulting in phenotypic diversification (McPhail 1994; Taylor and Bentzen 1993; Schluter and McPhail 1992, 1993; Foote et al. 1989). In sticklebacks (*Gasterosteus aculeatus* complex) from the Strait of Georgia, British Columbia, Canada, adaptive

radiation has produced pairs of sympatric species that have undergone parallel divergence in morphology, ecology, and reproductive behaviour (McPhail 1993, 1994; Schluter and McPhail 1992; Schluter and Nagel 1995; Nagel 1994). The sympatric species are not yet formally described and we refer to them as limnetic and benthic according to their habitat preference (McPhail 1993).

McPhail (1993, 1994; Schluter and McPhail 1992) has suggested that limnetics and benthics were established from two invasions of fresh water by marine threespine sticklebacks (we use "marine" to refer to populations that have fully marine, fully estuarine, or anadromous life histories) separated by approximately 1500 years (the double-invasion hypothesis). It has proved difficult, however, to resolve the evolutionary history of these species past a general statement

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R. Kassen, D. Schluter,¹ and J.D. McPhail. Department of Zoology and Centre for Biodiversity, University of British Columbia, Vancouver, BC V6T 1Z4, Canada (e-mail: schluter@bcu.ubc.ca).

¹ Author to whom all correspondence should be addressed.

that they became established in their present distribution sometime after the retreat of the Pleistocene glaciers approximately 12 000 years ago (McPhail 1993, 1994). The known geologic history of the Strait of Georgia region in southwestern British Columbia provides support for McPhail's double-invasion hypothesis (Mathews et al. 1970; Alley and Chatwin 1979; Armstrong et al. 1965).

Recent biochemical data (McPhail 1992) also lend support to the double-invasion hypothesis. McPhail screened 25 allozyme loci and calculated Nei's genetic distance between each sympatric form in Paxton Lake (Texada Island, British Columbia) and an anadromous population. The genetic distance between the limnetic and anadromous populations (0.016) was less than that between the benthic and anadromous populations (0.074), suggesting that the first colonizers of fresh water gave rise to the modern benthic species and the second marine incursion produced the limnetic species. However, because genetic distances are very small and based on just three polymorphic loci, the allozyme data provide only weak support for the double-invasion hypothesis. Moreover, preliminary data based on restriction fragment length polymorphisms of mitochondrial DNA are not consistent with the allozyme data (E. Taylor, personal communication), leaving the relationships between the sympatric species and anadromous sticklebacks highly uncertain. The historical picture is further complicated by the presence of solitary populations intermediate in morphology and resource use (Schluter and McPhail 1992) in neighbouring lakes, whose relationships to limnetics, benthics, and marine sticklebacks are untested. Here, we provide further evidence bearing on the evolutionary history of stickleback populations in the Strait of Georgia by examining their differences in a physiological trait, embryo hatchability in salt water.

Heuts (1947) has shown that anadromous sticklebacks hatch more young in salt water (approximately 32 ppt) than resident freshwater sticklebacks. We suggest that this may be explained by the gradual loss of alleles enabling salt-water survival after colonization of fresh water, where they no longer serve a function (and may even be detrimental). If this reasoning is correct, then we expect salt-water tolerance to be high in freshwater populations established recently, and lower in populations that have resided in fresh water for a longer time. Here we test the double-invasion hypothesis by applying this reasoning to order the dates of freshwater invasion in sticklebacks. If limnetic and benthic species are the result of freshwater invasions at different times, they should differ significantly in salinity tolerance (Schluter and McPhail 1992). Moreover, the species with the higher salt-water tolerance should be the more recent invader.

We present the results of an experiment that measured the salt-water tolerance of stickleback embryos from three lakes having sympatric species pairs, one lake containing a single species, and a nearby creek containing an anadromous population. Our goal was to contrast the salinity tolerance of limnetics and benthics and to compare it with that of a solitary population whose relationship to the sympatric species is unknown. We assumed that embryo hatchability in salt water was an indicator of viability in that environment, and hence a measure of salinity tolerance. Our results suggest that two freshwater invasions indeed took place: benthics and the solitary species invaded first, followed by limnetics

In addition, we compared mean egg size in the same populations plus an additional marine population to investigate whether this trait is also related to the amount of time a population has spent in fresh water. A preliminary reason for thinking this might be so is the widespread tendency for freshwater fish species to have larger eggs than their marine relatives (Kamler 1992; Holliday 1969; for sticklebacks specifically, see Snyder 1990 and Baker 1994). We found that benthics and the solitary species have larger eggs than limnetic and marine sticklebacks. We address the possibility that egg size may determine salt-water hatchability. We also discuss reasons for an evolutionary increase in egg size in fresh water. We conclude by presenting a modified version of the double-invasion hypothesis.

Methods

Study sites and geologic history

We used threespine sticklebacks from nine populations in the Strait of Georgia region of southwestern British Columbia. All are resident year-round in fresh water except the Cranby Creek population, which is anadromous and enters fresh water to breed, beginning in early June. The freshwater populations include two species (limnetics and benthics) each from Paxton, Priest, and Emily lakes on Texada Island and the single species from nearby Cranby Lake (McPhail 1993, 1994; Schluter and McPhail 1992). Priest and Emily lakes are in the same drainage; all other populations occur in separate drainages.

All freshwater populations except that of Jericho Beach were founded from the sea at the end of the Pleistocene (Fraser) glaciation between 13 000 and 10 000 years ago, when rising land levels isolated populations in small lakes along the coast (McPhail 1993, 1994; Schluter and McPhail 1992; Lavin and McPhail 1985; McPhail 1984). The Jericho Beach population is a marine population isolated in fresh water. It was founded approximately 25 years ago when a culvert connecting a lagoon to the sea collapsed and created Jericho Beach Pond (J. Lowden, Greater Vancouver Regional District Parks and Recreation, personal communication).

Collection, fertilization, and rearing of eggs

Fish were collected using minnow traps and dip nets. Each male or female was used only once when making crosses. Crossing was accomplished by removing and macerating the testes from a male and placing them in a petri dish partially filled with dechlorinated water. Eggs were then stripped from a gravid female and placed directly into the petri dish containing the testes. After 10 min, fertilized eggs were transported to plastic hatching cups with the bottom replaced with fine-mesh screen. Hatching cups were suspended in water above an air hose or air stone to create a current of well-oxygenated water around the egg mass. The hatched young were removed and counted using an eyedropper. Young were considered hatched when the tail became free from the yolk sac.

Salinity tolerance experiment

In May and June 1994 we collected sticklebacks from Paxton, Priest, Emily, and Cranby lakes and from the mouth of Cranby Creek. Water temperature was not controlled but remained

Fig. 1. Relative hatching success in salt water (mean \pm SE) for populations from Texada Island. All populations are resident in fresh water, except those in Cranby Creek, which is marine. n is the number of families.

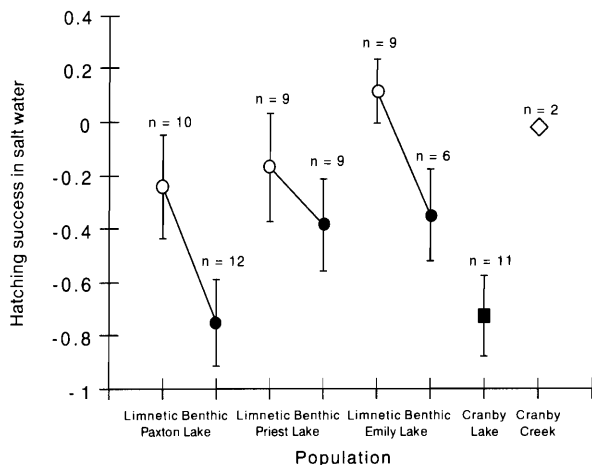


Table 1. Two-factor ANOVA examining relative hatchability in salt water between limnetics and benthics in Paxton, Priest, and Emily lakes.

Source	df	SS	MS	F	P
Lake	2	1.367	0.684	2.307	0.101
Species	1	2.799	2.799	9.445	0.003
Lake \times species	2	0.400	0.200	0.676	0.513
Error	51	15.115	0.296		

Note: Individual observations are family means. Lake was treated as a fixed effect. SS, sum of squares; MS, mean square.

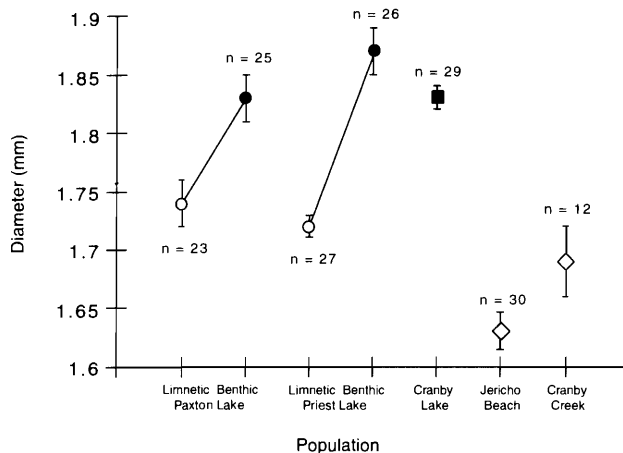
close to 17.5°C throughout most of the experiment (however, extreme temperatures of 15.5 and 19.0°C were also recorded). The eggs were exposed to the natural photoperiod (12 ± 1 h light and dark). The experiment was replicated and each replicate consisted of two 40-L tanks, one containing fresh water and the other salt water. All tanks were fitted with two box filters and multiple air hoses.

To compare the relative hatchability of fertilized eggs in salt water, we divided families into two batches and raised one in salt water and one in fresh water. We then calculated the proportion of young hatched in a single batch of eggs (young hatched/total eggs in batch) in salt water and in fresh water. Because data expressed as proportions form a binomial rather than a normal distribution (Zar 1984), proportions were angular transformed. We then subtracted the transformed proportion of young hatched in fresh water from the transformed proportion of young hatched in salt water, yielding a measure of relative salt-water hatchability for each family. If fewer than 10% of eggs raised in fresh water hatched, we assumed that the clutch was not successfully fertilized and the family was excluded from the analysis.

Egg size and morphological measurements

We collected gravid females from Paxton Lake (limnetics and benthics), Priest Lake (limnetics and benthics), Cranby Lake, Cranby Creek, and Jericho Beach Pond and stripped their eggs into petri dishes of dechlorinated fresh water. Before stripping the eggs, we measured the standard length

Fig. 2. Mean (\pm SE) egg diameter for six populations from the Strait of Georgia, B.C. All populations are resident in fresh water except those in Cranby Creek, which is marine. The Jericho Beach population was founded by marine sticklebacks 25 years ago. n is the number of families.



(mm) of each female. After counting all the eggs in the clutch, we removed 10 eggs at random and measured them to the nearest 0.02 mm using Aerospace vernier-scale calipers. To control for osmotic influences on egg diameter, we measured unfertilized eggs; fertilization causes the chorion to lift away from the egg, creating the perivitelline space, the size of which depends on the osmotic potential of the egg in relation to that of the ambient water (Alderdice 1988; Fuiman and Trojnar 1980).

Egg size is the mean egg diameter of the 10 eggs selected from each clutch. We did not correct egg diameter for female body size or number of eggs per clutch, since we did not find a significant association between egg diameter and the other two variables in five of the six populations tested ($r^2 \leq 0.12$, all $p \geq 0.08$). In the sixth population, Jericho Beach, egg diameter was negatively associated with clutch size ($r^2 = 0.34$, $P = 0.007$). We did not correct for this relationship because egg diameters from Jericho Beach fish were well below those of the other populations tested, so a correction made no difference to the results.

Results

Salinity tolerance experiment

Limnetics from Paxton, Priest, and Emily lakes hatched significantly more young in salt water than benthics (Fig. 1, Table 1) indicating that limnetic embryos were more tolerant of salt water than benthic embryos. The salt-water hatchability of limnetic embryos was high and similar to that of the anadromous embryos from Cranby Creek (Fig. 1). Cranby Lake embryos had a similar hatchability in salt water to the benthic populations (Fig. 1).

Egg size

In lakes containing a sympatric pair (Paxton, Priest) limnetics' eggs were significantly smaller than benthics' eggs (Fig. 2, Table 2). In Cranby Lake, egg size was similar to that of benthics from Priest and Paxton lakes. Jericho Beach had the smallest eggs and Cranby Creek had an egg size intermediate between limnetics and Jericho Beach. Of the two marine

Table 2. Two-factor ANOVA examining egg-size differences between limnetics and benthics in Paxton and Priest lakes.

Source	df	SS	MS	F	P
Lake	1	0.003	0.003	0.259	0.6116
Species	1	0.445	0.445	45.164	0.0001
Lake × species	1	0.019	0.019	1.957	0.1645
Error	118	1.164	0.01		

Note: Individual observations are family means. Lake was treated as a fixed effect. SS, sum of squares; MS, mean square.

populations, in Jericho Beach and Cranby Creek, the latter had the smallest sample size, therefore we do not consider it further.

Discussion

Hatchability and the double-invasion hypothesis

Our results support the double-invasion hypothesis. The relative salt-water hatchabilities for populations from Paxton, Priest, and Emily lakes (Fig. 1) indicate that in all three lakes, limnetics hatched significantly more young in salt water than benthics. If the physiological clock is even qualitatively reliable, the results imply that limnetics and benthics have been in fresh water for different lengths of time, and there were two invasions of fresh water by ancestral sticklebacks. Salt-water hatchability in limnetics was nearly as high as in the anadromous population. This suggests that the ancestors of limnetics invaded fresh water more recently than the ancestors of benthics. Although a sample size of two for the anadromous population is not by itself compelling evidence, we have no reason to believe that this result is inaccurate: our data are consistent with those of Heuts (1947) and a pilot experiment by R. Kassen (unpublished data), who found that marine sticklebacks have relative salt-water hatchabilities close to 0, indicating that these populations have high salinity tolerances. The solitary population from Cranby Lake hatched similar numbers of young to the benthic populations, suggesting that this population was formed during the first invasion. Assuming that salt-water hatchability decreases as the time since freshwater colonization increases, we suggest that (i) the double-invasion model is qualitatively supported; (ii) limnetic ancestors colonized fresh water after benthic ancestors; and (iii) the solitary population invaded at the same time as the benthic populations.

These data complement the results of McPhail's (1992) allozyme studies supporting a double-invasion scenario, where benthic ancestors invaded before limnetic ancestors. However, double invasion may not be the sole explanation for the pattern of salt-water tolerance that we observed; it is conceivable that limnetics, benthics, and the solitary species could have invaded fresh water at the same time, and our results indicate evolved differences among the species in salt-water hatchability or a correlated trait that are unrelated to the time since freshwater invasion. Further evidence from a wider range of traits is required before the double-invasion hypothesis can be definitively accepted or rejected.

Egg size and the double-invasion hypothesis

Limnetics had smaller eggs than both the solitary population from Cranby Lake and benthics, and these differences are

not due to differing salinities of the maternal environment during egg development: marine sticklebacks from Jericho Beach retain their small egg size when raised in fresh water (Fig. 2). If egg size in the Jericho Beach population is typical of the anadromous form shortly after freshwater invasion, this result implies that egg size has increased in all freshwater populations since freshwater invasion, and that limnetics are closer to the ancestral state than are benthics.

Although we have no prior evidence to suggest that egg size evolves in a clock-like fashion, these results are surprisingly congruent with the pattern observed for hatchability: limnetics and Cranby Creek fish, which have small eggs, hatch relatively more young in salt water than benthics and Cranby Lake fish, which have large eggs. One explanation for this pattern is that salt-water hatchability is in some way determined by egg size. For example, small eggs, having a larger surface-to-volume ratio than large eggs, may be more efficient at exchanging gases such as oxygen, which is scarcer in salt water than in fresh water of the same temperature. Data from Heuts (1947) and unpublished data of R. Kassen suggest that salt-water hatchability is maternally inherited, and since egg size is also a maternal trait, the above mechanism seems plausible.

Thus, the differences in salt-water hatchability we observed might simply be a correlated effect of an evolutionary increase in egg size in fresh water. However, this leaves open the question as to why the eggs of benthics and the solitary population are larger than those of limnetics. Conceivably, egg size is dependant on the time since colonization of fresh water (see also Baker 1994), in which case our results are still in accordance with the double-invasion hypothesis. But another question remains: why should selection favour the evolution of larger eggs in fresh water at all?

A number of hypotheses seem plausible, none of which can be ruled out. Larger young at emergence may be more adept at avoiding predators (Taylor and McPhail 1985), so differential predation between marine and freshwater populations may influence egg size (Pepin et al. 1987; Baker 1994). Larger eggs might also be favoured by limited juvenile dispersal in lakes, resulting in higher levels of intraspecific competition relative to anadromous populations that can disperse into the sea soon after hatching (Fleming and Gross 1990). Egg size may also be related to seasonal food availability (Bagenal 1971; Baker 1994). For example, if prey availability in lakes is seasonally variable, then larger eggs that produce larger young capable of surviving longer without food may be favoured. Temperature may also be important in determining egg size (Beacham and Murray 1985; Fleming and Gross 1990; but see Beacham and Murray 1993). If the optimum temperature for converting yolk to embryonic tissue is low, as it is for salmon (Beacham and Murray 1985), then a cool marine environment during the breeding season favours smaller eggs.

The double-invasion hypothesis revisited

The differences in egg size and salt-water hatching success between limnetics and benthics are consistent with McPhail's (1992) allozyme data; limnetics invaded fresh water more recently and are therefore more closely related to present-day marine species than are benthics. However, the differences in egg size and hatching success were larger than we expected given our present understanding of the double-invasion hypoth-

esis (McPhail 1993); 1500 years between invasions seems too brief a period to produce the differences observed.

It may be that the differences in egg size and hatchability accurately reflect the different times of freshwater invasion, but that benthics have been in fresh water for longer than the 10 000 – 12 000 years since deglaciation. In this scenario, limnetics would have invaded freshwater from the sea 10 000 – 12 000 years ago but benthics would have resided in fresh water for many years prior to the retreat of the glaciers. The differences in egg size and salt-water hatchability, therefore, may reflect the amount of time populations have spent in fresh water, but not the length of time their present geographic distributions have existed.

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