2012

Salinity Preference of Alaskan Threespine Stickleback: Test for Divergence in Halotaxis between Ancestral and Landlocked Populations

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Biological Sciences
May 2012

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An undergraduate Honors Scholar Thesis in partial satisfaction of the University Scholar Project: *Salinity Preference and Microevolutionary Changes in Osmoregulatory Physiology of Landlocked Threespine Sticklebacks*

Abstract: Glacial retreat during the Pleistocene caused landlocking of anadromous Alaskan threespine stickleback, *Gasterosteus aculeatus*, furnishing a natural 'experiment' in osmoregulatory divergence. The objective of this study was to assess the effects of individual acclimation and population divergence on salinity preference. Full-sibling families of marine, anadromous, and freshwater-landlocked populations of stickleback were reared in common environments until 3 weeks post-hatch, then were split and acclimated to low or high salinity. At 6 to 8 weeks of age the six experimental groups were tested for salinity preference in a tank that offers fish a choice of compartments with different salinities arranged in a gradient from fresh to sea water. We observed significant population and acclimation effects. Anadromous fish preferred sea water and avoided fresh water, whether acclimated to low or high salinity. Landlocked fish showed a strong acclimation effect, avoiding salt water when acclimated to fresh and avoiding freshwater when acclimated to salt, while showing no preference for their acclimation salinity. Fish from the marine population showed little preference for fresh or sea water regardless of acclimation salinity. After restriction to fresh water for more than five thousand generations, landlocked fish have evolved weaker preferences in response to a salinity gradient compared to their anadromous ancestors.

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Introduction:

Evolutionary theory predicts loss of adaptive plasticity after periods of environmental stasis (Masel et al. 2007) through "relaxed selection," defined by Lahti et al. (2009) as when "environmental change… eliminates or weakens a source of selection that was formerly important for the maintenance of a particular trait." Trait loss was traditionally assumed to be the result of selective neutrality (Fong et al. 1995), however there is mounting evidence that suggests trait loss can be due to selection *against* costly traits after new environments no longer require their maintenance (reviewed by Lahti et al. 2009). When plasticity is itself considered a trait, these mechanisms result in canalization. Canalization is known to cause genetic assimilation of a formerly plastic trait when an environmentally induced phenotype becomes selected for (Waddington 1953). Adaptive osmoregulatory plasticity in landlocked, ancestrally euryhaline threespine stickleback, is therefore predicted to be lost through relaxed selection, since it has been 12,000 years since landlocking occurred (Barrett et al. 2009). Pilot studies performed by Divino and Schultz (2010, 2011) suggest that freshwater landlocked populations of Alaskan threespine stickleback have lost some plasticity in osmoregulatory ability. However, landlocked stickleback did show negligible mortality in full salt water (35 ppt). This suggests that over the >4,000 generations of adaptation to freshwater environments, osmoregulatory physiology of landlocked stickleback has not been significantly canalized nor has experienced significant relaxed selection, i.e. there remain no clear physiological barriers to inhabiting a marine environment. What, then, prevents landlocked stickleback from migrating to sea, as their ancestors once did?

The answer is obvious: geographical/physical barriers (land). Consider the hypothetical question, then: if these geographical/physical barriers were removed, would landlocked

stickleback, which retain the ability to physiologically persist in saltwater, choose to migrate to salt water? In other words, have landlocked stickleback evolved a behavior in the form of a salinity preference that might act as a barrier hindering re-invasion of saltwater?

Since it has been found that relaxed selection has caused minor loss in osmoregulatory plasticity in landlocked stickleback populations, and since behavior in the form of salinity preference is likely coupled with salinity tolerance (the physiological ability to handle environments of certain salinities), one would expect that freshwater landlocked populations have experienced a loss in saltwater preference. Likewise, since freshwater landlocked stickleback have been unable to exhibit halotaxis (directional movement in response to a salinity gradient) because no salinity gradient exists in the freshwater ponds they inhabit, one would expect preference to decay due to relaxed selection. In this study, divergence in salinity preference is tested between derived landlocked stickleback and their ancestral anadromous and marine relatives, yielding insights into adaptive divergence and the evolutionary history of freshwater invasions.

Osmoregulation and Freshwater Invasion by Fishes

A critical moment in evolutionary history takes us back 400 million years ago to the Devonian Period, when wide-scale invasion of freshwater environments led to a radiation of fish species. How did this epic "Devonian swim upstream," along with subsequent freshwater invasions, occur? Essentially opposite osmoregulatory mechanisms are needed for survival in fresh and salt water, perhaps a testament to why entire taxa, for example the phylum Echinodermata, have been unable to cross the salt boundary into freshwater.

Almost all extant fishes are osmoregulators (Evans and Claiborne 2006). An osmoregulator adjusts its internal osmolality to maintain homeostasis of blood solute concentration. Most osmoregulating fishes maintain an internal osmolality of about 300-400 mOsm/L independent of external solute concentrations (Krogh 1939 and Smith 1932). Saltwater fishes must fight dehydration by excreting excess ions and retaining water, while fishes living in freshwater must do the opposite; they must retain solutes and discharge excess water to prevent inundation (Krogh 1939). Osmoregulation in both environments occurs at the gills, gut, kidney, and epidermis. The polarity of the physiological mechanisms involved with maintaining internal osmotic homeostasis in these two opposite environments makes euryhalinity a remarkable characteristic.

Euryhalinity can be defined in two ways. In one sense, a euryhaline fish is one that experiences relatively large fluctuations in salinity over its lifetime, such as diadromous or estuarine fish (distribution-euryhaline). In another sense, euryhalinity is the physiological ability to osmoregulate in a wide range of salinities, which must be determined experimentally (physiologically euryhaline). The distinction between these definitions can be understood with consideration of landlocked stickleback as an example. In terms of the first definition, landlocked stickleback are not euryhaline, but stenohaline, since they do not experience fluctuations in salinity through their life. However, they are euryhaline by the second definition, since they have the physiological capacity for osmoregulation in a large range of salinities.

Another form of euryhalinity that is distinct from traditional meanings is "evolutionary euryhalinity." Hutchinson (1960) describes a taxon of animal as demonstrating evolutionary euryhalinity if members of its subtaxa inhabit fresh and salt water. After a broad literature search, Schultz and McCormick (2012) suggest that many fish taxa exhibit evolutionary

euryhalinity, and that multiple and repeated freshwater/saltwater transitions in evolutionary history characterize many clades.

The importance of freshwater invasions to the diversity of fishes as well as the evolution of first terrestrial vertebrate life is paramount. Freshwater invasion eliminates connectivity and gene flow between populations relatively rapidly, leading to adaptive radiations, similar to situations studied in island biogeography. Limited gene flow among freshwater fish populations due to the frequent presence of geographic barriers in freshwater systems has undoubtedly led to the diversity of extant freshwater bony fishes, which make up 41% of all known bony fishes, despite the large majority of Earth's surface and volumetric waters being salty (Berra 2007).

Freshwater invasions can occur in two ways: fish can either become landlocked by a physical barrier or they can evolve a preference for freshwater first, causing them to exist in only freshwater. In other words, freshwater invasions lead to barriers to the marine environment that can be behavioral or physical (physiological euryhalinity is *prerequisite* for freshwater invasion, so physiological barriers to freshwater environments are not considered). However, at least once in the evolution of fishes, since today there exist populations of fish, and specifically stickleback (Honma and Tamura, 1984; Jones et al., 2006), with no physical/geographical barriers to seaward migration, they must have either 1) evolved a preference for freshwater first, allowing them to migrate upstream, or 2) become landlocked by geographical/physical barriers and lost their preference for salt water, thereafter inhabiting river systems that had no physical barriers to the marine environment. The likely case, given the number of repeated invasion events through time, is that both of these situations have occurred.

How fast does salinity preference evolve after landlocking? Does preference for environmental salinity even evolve in the presence of environmental stasis with respect to

salinity? Or is it that only distribution-euryhaline fish have evolved strong preference for salinity?

Since we know the approximate length of time since stickleback landlocking, and there are no physiological constraints disallowing landlocked stickleback to survive in saltwater, we can examine how quickly saltwater preference is lost after landlocking of anadromous fish by experimentally determining salinity preference in derived landlocked versus ancestral anadromous stickleback.

Review of Salinity Preference

Salinity preference of fishes was first studied in 1915 (Wells, 1915), and has since been studied in a variety of species. A nonexhaustive literature search suggested that about 40 salinity preference studies have been performed on fishes, with at least another 40 on a diversity of other organisms. Other organisms studied include most often crabs, but also frogs, snakes, various insects, amphipods, shrimps, diatoms, and oligochaetes.

However, many of these salinity preference studies do not involve behavioral preference. For example, diatom salinity preference was determined as the salt conditions at which growth was greatest (Underwood 2000, Chowdhury et al. 2008). In other studies, preference was considered the salinity with low mortality (Hoback et al. 2000 with tiger beetles, Cort 1993 with freshwater prawn larvae), often referred to elsewhere as tolerance. Many other salinity preference studies infer behavioral salinity preference by analyzing geographic distribution of organisms relative to salinity (Scott 1982 with Scotian Shelf fish, Roberts et al 1997 with mosquito larvae, and Calliari 2007 with mysids). However, separating salinity effects from other biological, physical, and chemical factors is not possible in such studies (Serrano 2010). Here we

consider salinity preference as a behavior, which must be determined experimentally by testing an organism's response to salinity.

Several types of apparatus have been employed for the study of behavioral response to a salinity gradient. Each design reflects the organism of study. For example, studying preference in a frog allows for chambers of water with various salinities to be connected via above-water bridges since frogs can move extra-aquatically. However, in exclusively aquatic organisms, such as fishes, preference experiments need be more creative. Below we outline the general designs used in behavioral preference tests of various organisms.

Preference studies in frogs have consisted of at least two separate chambers of water with varying salinity that can be experienced by hopping over a divider (Davenport 1997)(Design 1). Other studies for which a terrestrial bridge between salt solutions was employed include a design with two troughs connected at a central shallow peak that is barely outside of the water and traversable by the organism. This design was used in both oligochaetes (Jannson 1962) (Design 2) and amphipods (McLusky 1970) (Design 3). Modifications of this design used to study crab preference used several troughs for a more continuous gradient of salinities (Ameyaw-Akumfi and Naylor 1987, McGaw and Naylor 1991).

In other cases where the organism cannot exit the water, most designs make use of the fact that salt water is denser than fresh water. This difference in density allows different salt solutions to stratify with surprisingly minimal mixing. Such designs are themselves diverse.

In the simplest example of this group of designs, a one tank system was used to study the preference of crab zoeae based on the salinity strata at which they aggregated (Capaldo 1993) (Design 4). This design consists of one cylinder with a vertical gradient from the densest salty

water at the bottom to fresh water at the top. If the tank is not disturbed once filled, then the vertical salinity gradient remains surprisingly stable.

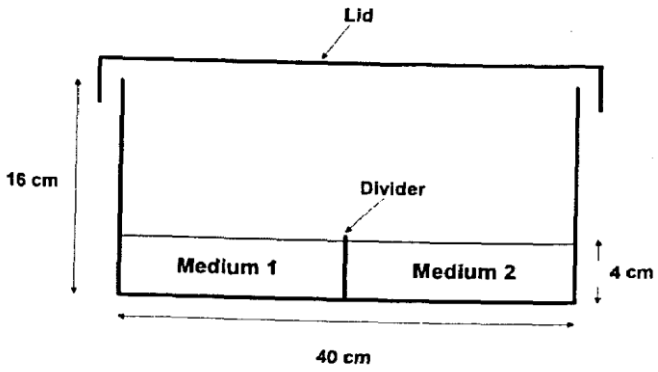
Early versions of the widely-used Staaland device attempted to create a continuous gradient horizontally, however these designs had the issue of high amounts of interchamber mixing (Baggerman 1957) (Design 5). Other more commonly employed designs for fish include variations of the Staaland (1969) (Design 6) (many studies refer to the Staaland-modified Fivizzani and Spieler (1978) device) type device which all use a horizontal gradient that is highly resistant to interchamber mixing of salt solutions. By designing a several-chamber tank in which adjacent chambers are connected by a less dense fresher water layer on top, the gradient remains stable. In order for an organism to traverse a multi-chamber Staaland-type tank, it must swim up and down repeatedly. These types of tank are the most popular since they allow for a gradient of several different salinities depending on how many chambers are used. Staaland's original design included eight chambers, but subsequent modifications have used as few as three. A well-designed study on largemouth bass used five chambers (Meador 1989) and another study to assess the difference in preference between closely related estuarine spot and croaker used six chambers (Moser & Gerry 1989). Some studies have discovered an edge effect in Stalaand-type devices that may be species and/or tank specific. Although these edge effects can usually be addressed with carefully designed control trials, Kolsch (2010) (Design 7) designed a circular modified-Staaland tank that eliminated the edge effect for the study of salinity preference in beetles.

A few more notable designs that do not use the density differential to maintain gradients include a two choice design by Jury et al. (1994) in which lobsters were placed in a tank that had a central partition which separated salt solutions, and a submerged PVC pipe tunnel between the

partition. This design worked well in their study since they were testing salinities of 25 ppt and 30 ppt, which are similar. Such designs would not work for a study between 0 ppt fresh water and 30 ppt salt water because a little mixing would cause a great change in the freshwater salinity.

Another design includes a two-choice flow-through tank where water of two salinities is simultaneously run down two convergent flow-ways. A fish is released at the bottom of the flow-way and can choose which tank it wants to swim towards based on the different salinities of the flowing water. This design has been successfully used in red drum (Parkyn 2002) and various galaxiids (Hale 2008) (Design 8).

Another notable, yet complicated design is called an electronic shuttlebox in which real-time decisions by fish are used to change the salinity in either of two tanks. For example, if a fish traverses a channel from a low salt tank to a high salt tank, the electronic shuttlebox system will detect that choice and begin adding salt to the lower-salt tank. This allows for a sort of organism-dictated fine-tuned preference. This design was modified from a temperature preference study apparatus by Schurmann et al. (1991) and was used to study salinity preference by Serrano et al. (2010) in grey snapper. Such complicated designs are expensive and their results are often difficult to interpret.



Design 1: Davenport 1997, Simple preference tank for frogs

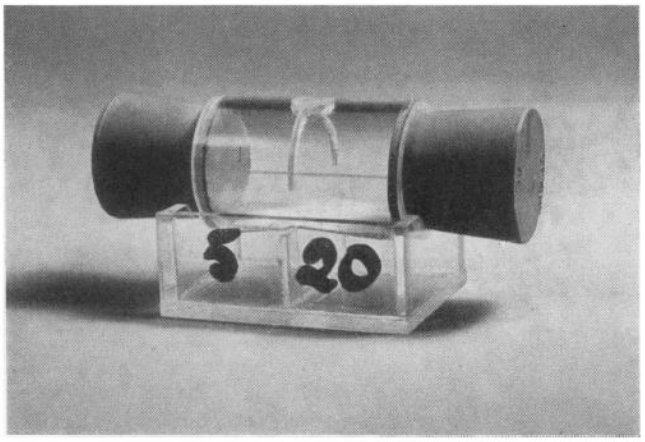


Fig. 1. Alternative chamber for salinity experiments. The chamber with rubber stoppers resting in the box.

Design 2: Jannson 1962, Two chamber shallow preference tank for oligochaetes

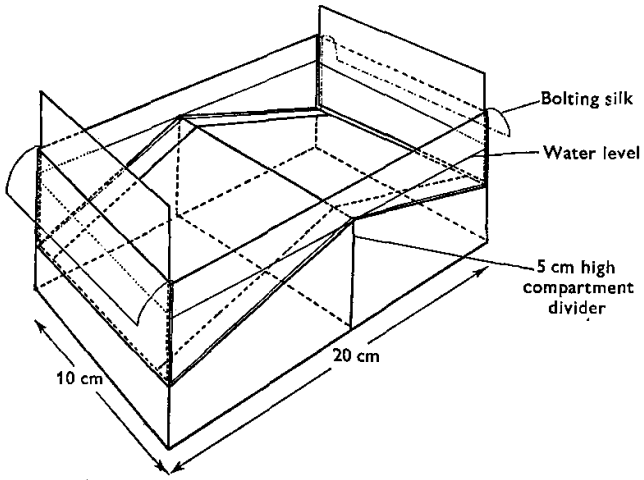


Fig. 1. Apparatus used for salinity preference experiments.

Design 3: McLusky 1970, Two chamber design for amphipods

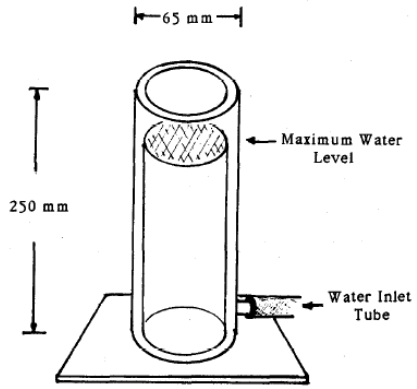


Fig. 1. Diagram of the plastic column used in tests to determine salinity preferences for the zoeae of the three test species. Water of varying salinity was introduced through a tube attached at the base of the column. Zoeae were observed to concentrate in dense bands, which were salinity dependent.

Design 4: Capaldo 1993, Vertical salinity gradient for crab zoeae

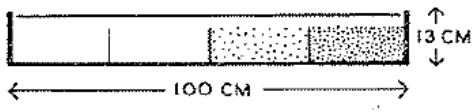


Fig. 9. Trough used for salinity tests. Compartments on the left: fresh water; compartments on the right: salt water. The density of the dots indicates the concentration of salt water. For further explanation see text.

Design 5: Baggerman 1957,Freshwater-overflow trough design

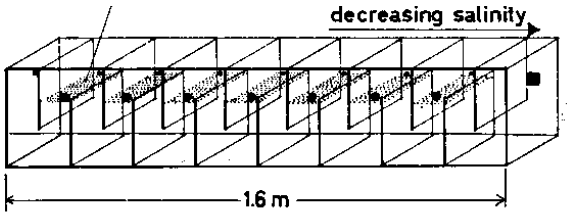
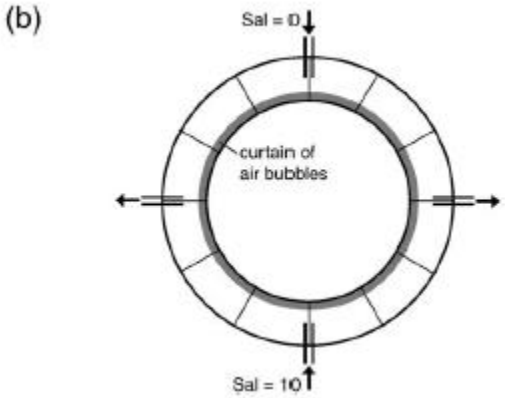
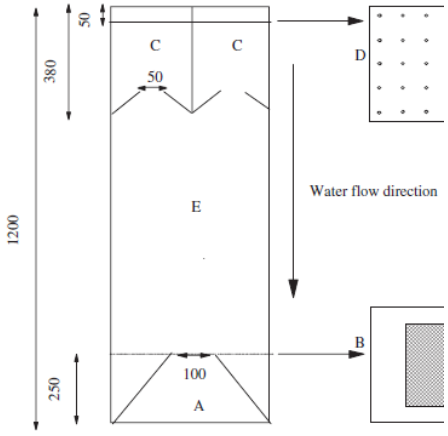


FIG. 1. The device (wall-thickness: 6 mm Plexiglass).

Design 6: Staaland 1969, Multichamber continuous gradient



Design 7: Kolsch 2010, Circular recreation of Staaland device



Design 8: Hale 2008, Two-choice floway design

Factors affecting salinity preference in different fish species have been discovered experimentally, such as age, temperature, acclimation salinity (Fritz 1974, Baggerman 1957), reproductive status (Baggerman 1957), hormonal status (Audet 1985, Baggerman 1957), parasitic infection (Webster 2007), and dark/light ratio (Spieler 1976). Most of these studies examine salinity preference to better understand either species distributions (Buck 2011, Serrano 2010, Parkyn 2002, Moser 1989, Fritz 1974, for example) or ontogenetic shifts in salinity preference in diadromous fish such as eels, galaxiids, salmon, and sticklebacks (Cook 2010, Edeline 2006, Crean 2005, McInerney 1963, Baggerman 1957). In this study we examine salinity preference between different populations acclimated to different salinities to test for population and acclimation effects.

Stickleback and Salinity Preference

To date, relatively few studies have been published examining differences between stickleback populations in traits related to osmoregulation, despite that the most fundamental change after landlocking is reduced environmental salinity. Prior research on freshwater stickleback has focused on determining morphological and ecological evolution post-landlocking (Bell and Foster 1994). Current detailed studies are taking place on the divergence in osmoregulatory physiology between stickleback populations (Divino and Schultz). In conjunction with these studies we examine in this study the divergence in salinity preference of the same populations of stickleback.

Salinity preference experiments have been performed on sticklebacks in the past. The earliest and most extensive study on stickleback salinity preference was performed by Baggerman in 1957. Her seemingly countless experiments resulted in a publication of about 200

pages describing salinity preference experiments aimed at elucidating effects on the timing of breeding and migration in threespine stickleback. She studied the effects of light/dark ratio, age, reproductive maturity, light intensity, and the various interactions of these traits on salinity preference. She did so using the previously described Design 5. The overall conclusion of her extensive study was that the "timing of the reproductive season is achieved by a very delicate interaction between intrinsic and external factors." She was looking to characterize factors affecting migration times in live-caught threespine stickleback with no intentions of discerning evolved interpopulation differences nor addressing acclimation effects.

Years later, several studies were performed aimed at defining interspecific differences in stickleback salinity preference. Various combinations of commonly-reared threespine, fourspine, ninespine, fifteenspine and black-spotted stickleback were tested in a Staaland tank in these studies. Campeau (1984) found different preferences and tolerances between the threespine and black-spotted stickleback that corresponded with field distributions of the species. Audet (1984) found hormonal (prolactin and cortisol) effects of salinity preference that were similar between threespine and fourspine stickleback. Audet (1985) found different salinity preferences that corresponded to field distributions of threespine, ninespine, fifteenspine, and black-spotted stickleback. Audet (1986) found effects of photoperiod and temperature acclimation on salinity preference in threespine, fourspine, and black spotted stickleback, but not in ninespine stickleback.

One more study on stickleback preference was performed by Barrett et al. (2009) that aimed to study the genotype of threepine stickleback in relation to the armor-related gene called ectodysplasin (Eda). They found no association of the low-armor eda allele with freshwater preference, despite the low armor allele being present in most freshwater threespine stickleback

populations. Their methods, however, involved a two chamber fresh/salt spillover design (like Design 5), which did not allow fish to have a gradient of salinities and likely caused the freshwater side to become salty fairly quickly.

To our knowledge our study is the first to examine intraspecific differences in salinity preference comparing landlocked freshwater ecotypes with their anadromous and marine ancestors. Using a four-chamber modified Staaland device, the salinity preference of commonly reared ancestral and landlocked threespine stickleback was tested at different salinity acclimations, to assess osmoregulatory behavioral divergence.

Hypotheses

Divergence between landlocked and ancestral anadromous and marine populations of stickleback has been widely reported in traits related to morphology and ecology. Likewise, we expect divergence between these populations in osmoregulatory physiology given the stasis of environmental salinity experienced by landlocked threespine stickleback over the last 12,000 years, and with it, divergence in salinity preference. We hypothesized that there would be interpopulation differences in salinity preference. More specifically, we hypothesized that landlocked fish would show relatively weaker preferences for saltwater (due to relaxed selection on osmoregulatory ability in saltwater or canalization of osmoregulatory plasticity) due to the predicted coupling of halotaxis behavior and physiological ability. We also hypothesized that we would find evidence for the loss of the behavioral trait of halotaxis in landlocked populations since it is not required for survival in stenohaline environments. We also expected salinity preference to vary based on acclimation salinity, so we tested these fish acclimated to two different salinities.

Methods:

Embryo Acquisition

Full sibling families of threespine stickleback (*Gasterosteus aculeatus*) were spawned in vitro from pairs of fish captured at each of three locations (Figure 1): Resurrection Bay (Marine, "Bay"), Rabbit Slough (Anadromous, "RS"), and Frog Lake (Freshwater, "LK"),Alaska, on June 7, 2011 by collaborators from Clark University. At these locations adult fish were caught using a combination of the following gear: 1/8-inch mesh metal minnow traps, 1/4-inch mesh metal minnow traps, hand-nets, and 10-ft x 6-ftminnow seine. At each location pairs of fish were captured and euthanized. Eggs were stripped from females and placed in a Petri dish while the males' testes were removed and rinsed in sterile water, macerated, and added to eggs for 15 min. The embryos were then rinsed in sterile water and put in embryo medium (~0.8ppt). These zygotes were shipped from Alaska to UConn while kept chilled on ice in a sterile cylinder with embryo medium.

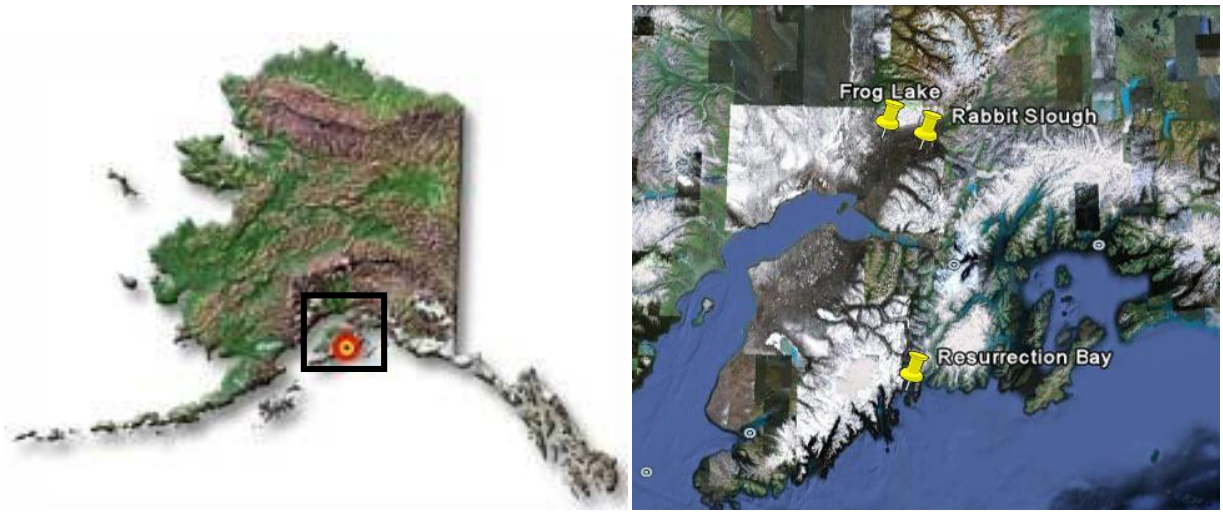


Figure 1: The location of sample sites where adult sticklebacks were captured and spawned. Resurrection Bay (60.12178 N, 149.40510 W) is a marine location, Rabbit Slough (61.53590 N, 149.25305 W) is a freshwater stream that drains into Cook Inlet, and Frog Lake (61°36'51.24" N, 149°43'07.30 W) is a small, isolated freshwater lake.

Rearing

On June 9, 2011 the embryos arrived at UConn. Embryo masses were separated and counted into groups of 25 individuals. These groups were each placed into 100mm petri dishes filled with 0.5 ppt water, verified using a YSI conductivity sonde, made from Instant Ocean® and reverse osmosis (RO) water (salt solutions were made in this manner throughout this study). Water was changed in each dish every other day and mortalities were removed every day. The fish room was kept on a schedule of 14 hours of light/10 hours dark and at 17-22 C throughout the study.

Table 1: Percent survival of embryos to hatching by family.

Family	Cross	Clutch Size	Total Hatched	Percent Hatched
Lake1	LKxLK	206	40	19.42
Lake2	LKxLK	191	7	3.66
Lake3	LKxLK	184	80	43.48
Anad1	RSxRS	172	158	91.86
Anad2	RSxRS	204	185	90.69
Anad3	RSxRS	250	212	84.80
Marine1	BayxBay	150	51	34.00
Marine2	BayxBay	267	182	68.16
Marine3	BayxBay	131	0	0.00
Marine4	BayxBay	250	0	0.00

Fish hatched 8-10 days post-fertilization. Families had variable hatching percentages (Table 1). Chorions were removed after hatching and the water was changed to 3.0 ppt in all dishes to minimize fungal infection. Yolk-sac stage lasted ~2 days during which small amounts of live brine shrimp (*Artemia* sp.) were introduced into the dishes. Fish generally first fed on brine shrimp 3 days after hatching, once yolks were fully absorbed. Fish were fed brine shrimp twice daily for the remainder of this study, supplemented by freeze-dried copepods at older ages.

Seven days post-hatch, fry were transferred to quart-sized glass jars filled with 250 mL of 3.0 ppt water at densities of 17-30 fish per jar. Jar water was changed every three days at volumes >50%. After seven days in jars, the fish were transferred to 10 gallon tanks filled with water of 3.0 ppt. Each tank contained a mixture of all families within a population. Tank water was periodically checked for levels of ammonium and nitrates, and >50 percent of tank water was replaced approximately once a week throughout this study. Tanks each had filters that ran during nights so as not to suck in brine shrimp during the day. Each aquarium contained BioBricks® for maintaining denitrifying bacteria populations, surf clam shells from Cape Cod, Massachusetts to potentially provide extra calcium and trace elements that are sometimes absent from artificial salt mixes, and artificial macrophytes.

Table 2: Background information; a) timeline b) population census through time c) approximate density of fish per tank after the start of acclimation and before the start of preference trials.

a)Timeline

Date	Event
7-Jun	Fertilization
9-Jun	Egg arrival to UConn
15-Jun	First Hatching
17-Jun	Change water to 3 ppt
18-Jun	First Feeding
19-Jun	Last Hatching
22-Jun	Put into jars
30-Jun	Mixed families, put into tanks
7-Jul	Pops Split, acclimation begins
14-Jul	Acclimation ends
5-Aug	Salinity Pref trials begin
18-Aug	Salinity Pref trials end

b) Population Census

Date	Anad.	Lake	Bay
9-Jun	626	581	798
15-Jun	578	155	287
21-Jun	555	127	233
29-Jun	543	124	230
7-Jul	529	120	223
14-Jul	524	119	223

c) Fish Density

Group	Density /Tank	# Tanks	Approx Total Fish
Anad Hi	100	3	300
Anad Lo	110	2	220
Bay Hi	110	1	110
Bay Lo	110	1	110
Lake Hi	60	1	60
Lake Lo	60	1	60

Each of the three mixed-family population tanks were evenly split 3 weeks post-hatch into acclimation groups of Hi (30.0ppt) and Lo (1.0 ppt) salinity. Over the next week the salinity was gradually decreased in Lo treatment tanks and gradually increased in Hi treatment tanks from the original rearing medium of 3.0 ppt to the acclimation salinities (Figure 2). Due to higher densities in certain populations, caused by variable mortality by population, the Anadromous Lo tank was haphazardly split into two tanks and the Anadromous Hi tank was haphazardly split into three tanks (Table 2b, 2c). Therefore there were nine rearing tanks in total, comprised of five anadromous fish tanks, two lake fish tanks, and two bay fish tanks. See Table 2a for a rearing timeline.

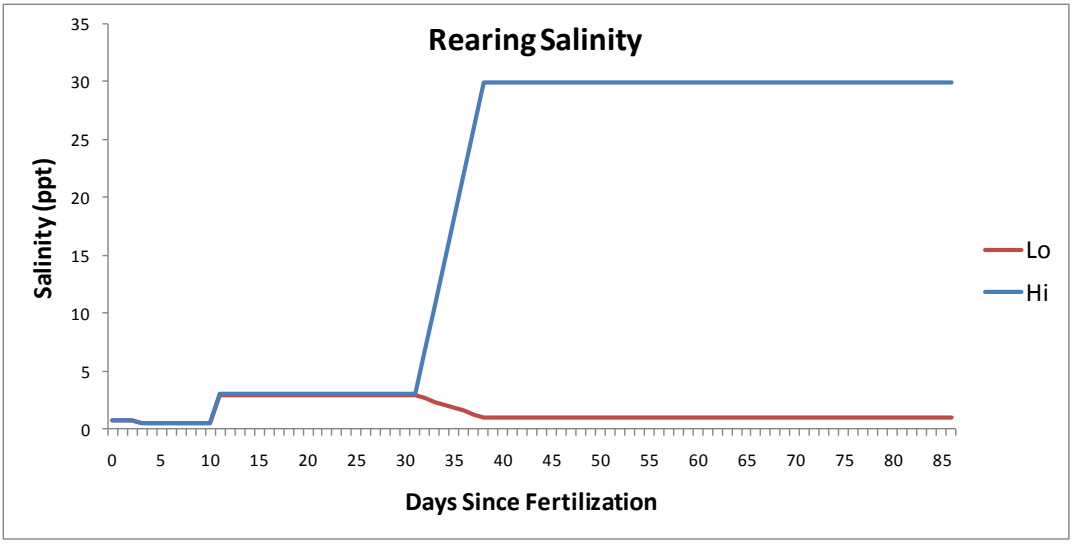


Figure 2: Rearing salinity over the duration of the experiment for the two acclimation groups

Unfortunately, the Bay Lo rearing tank experienced a near-entire die-out on August third. Other Bay fish from a different family and a slightly different rearing history were promptly acclimated to the Lo salinity (1.0 ppt) and used in place of the dead fish. The group of fish used came from a mixture of Bay families collected at the same location of those tested at the Hi salinity, but they had been reared entirely at 0.5 ppt in a very dense tank, and were a 3 days older

than the rest of the fish tested. The practical repercussion of this is that Bay Lo fish were not from the same clutches as Bay Hi fish, and they had slightly different rearing histories.

Salinity Preference Trials

At about five weeks post-hatch five fish per population acclimation were euthanized for gill tissue mRNA samples (for assays not described in this thesis). For the remainder of week five and week six, "pilot" preference trials were run to determine an optimal experimental design. These early preference trials were excluded from the experiment, but used 40 fish from both the Bay and Anadromous populations and 20 fish from the Lake population, decreasing the number of available fish (Table 3).

Table 3: The number of fish available for preference testing (after pilot trials and gill samples were taken) for each of the six experimental groups.

		Acclimation	
		Hi	**Lo**
Population	**Lake**	46	42
	Anad	265	199
	Bay	88	82

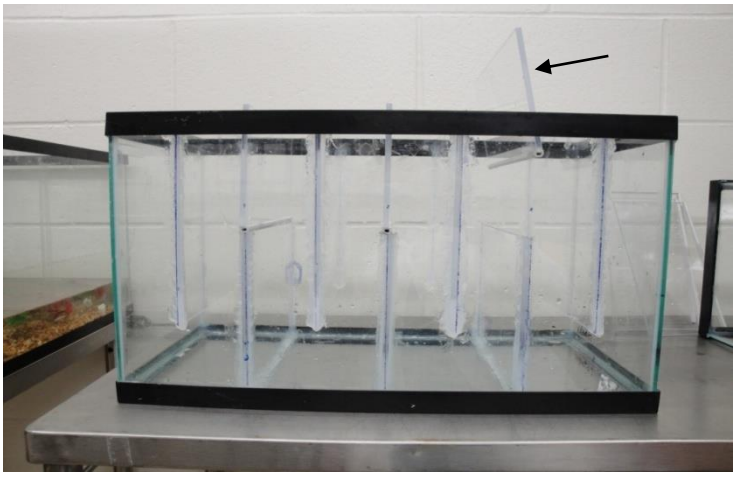


Figure 3: A picture of one of the four identical modified Staaland devices used in salinity preference trials, made from 10 gallon aquaria and glued plexi glass panels. Removable dividers (highlighted) allowed filling of the tank with minimal interchamber mixing.

At seven weeks post-hatch we began testing for salinity preference. Four identical modified Staaland type devices made from ten gallon aquaria were used. These devices maintained a gradient of four different salinities but allowed fish to easily traverse the tank. Water input in chambers one to four was pure RO water, 1.0ppt, 3.0ppt, and 30.0ppt, respectively (Figure 3,4).Salt solutions were made one day prior to use to ensure each separate solution was at room temperature and to ensure that all artificial salt was dissolved. After chamber dividers were removed, some mixing occurred causing the gradient to become 0.1-0.4 ppt, 1.0-1.8 ppt, 8.0-11.6 ppt, and 27.0-28.6 ppt, representing freshwater, Lo acclimation/brackish water, isotonic water, and saltwater/Hi acclimation water respectively (Figure 4). Preference tank water was changed every day. A black blind was created completely surrounding each tank (except on top) with slit peep-holes so that observations could be made.

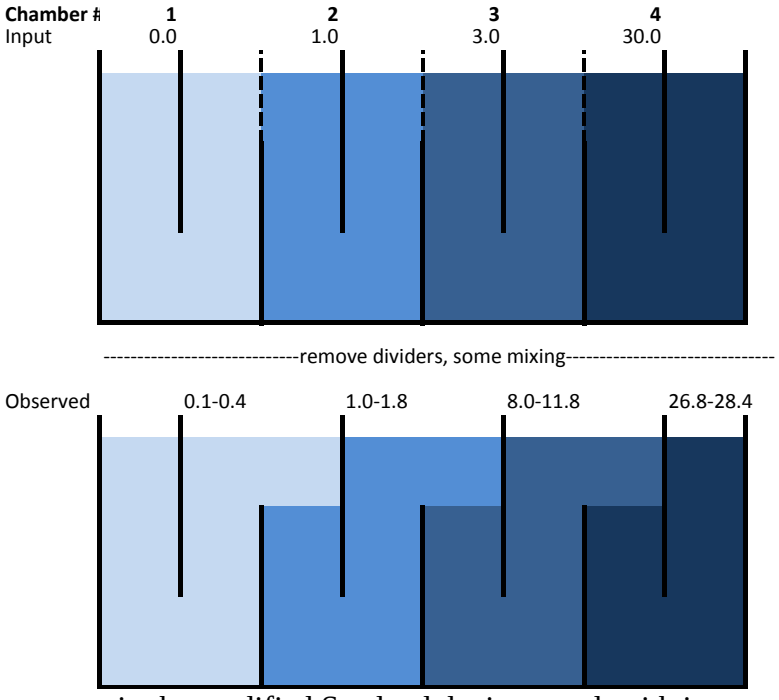


Figure 4: The gradient setup in the modified Staaland devices used, with input salinity and observed salinity in ppt. Tank chambers were filled with salt solutions while the dividers were in place, keeping the four chambers separate. After each of the chambers were filled the dividers

were removed and minimal mixing occurred, resulting in the final gradient ranges shown above, as measured at the end of each preference trial.

Two trials were run per day. Each trial simultaneously tested both Hi and Lo acclimations of a population in both control and gradient tanks (Figure 5). Control tanks consisted of homogenous (non-gradient) input at the acclimation salinity of the fish being tested (1.0 for Lo acclimation, and 30.0 for Hi acclimation).

Each tank tested five fish per trial. The five fish were placed in the center chamber at the beginning of each trial. Trials lasted 3 hours. For the first hour, fish location was noted every three minutes. For the second and third hours fish location was noted every ten minutes.

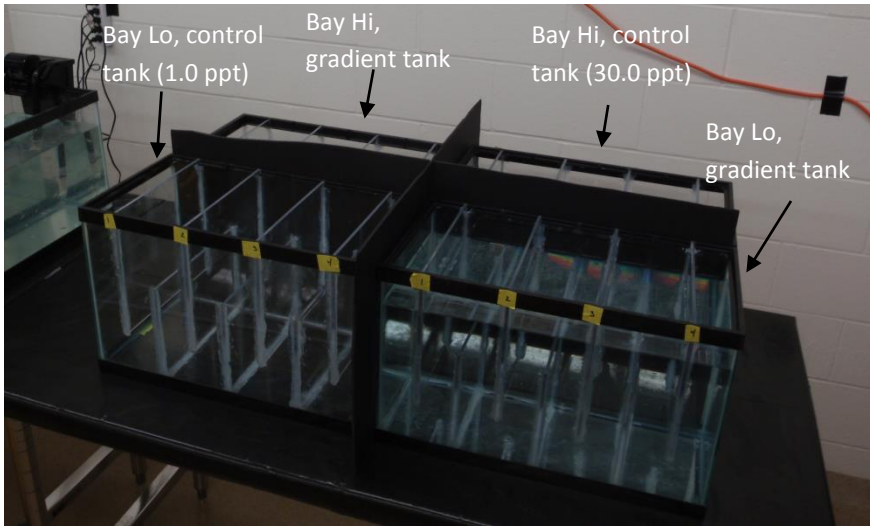


Figure 5: The four-tank experimental setup for a Bay population trial as an example. Lo/Hi refers to the acclimation salinity. Control tanks are filled with water of the acclimation salinity and the gradient tanks are filled as described in Figure 4. *Outer blind with slit peep-holes used when scoring fish positions during a trial is not shown.

Before the start of each trial, the five fish for each of the four tanks were photographed in a weigh boat containing a standard ruler. Length measurements were later taken from the photos using ImageJ freeware.

Data from the first sixty minutes of each trial were not scored for salinity preference but were used for a measure of fish activity. This period allowed the fish time to explore the tanks and experience the different salinities present in each chamber. Only the last two hours of each trial were analyzed for preference.

Eight trials were run per population, requiring a total of 160 fish per population. Since there were only 88 Lake fish available (Table 3), Lake fish from the first four trials were reused for the second four trials. With this exception, all fish were euthanized with an overdose of the anesthetic tricaine methanesulfonate (MS-222) immediately after a preference trial.

Tank assignments (control and gradient, Hi and Lo) were changed from day to day so that each of the four tanks used served an equal number of times as control or gradient, and Hi or Lo tank.

Salinity Preference Analysis

Since each ten minute timepoint was not independent within a trial, each two hour trial was treated as an observation. To achieve a single observation from each trial, proportional chamber use was calculated per trial for all of the gradient trials. For this the number of fish observations in a given chamber was summed over a whole trial. The sum per chamber was taken as a proportion of the total number of fish observations per trial.

$$\lambda = \frac{S}{T}$$

Equation 1: Proportional chamber use per trial, λ, is equal to the number of fish observations for a given chamber, S, divided by the total number of fish observations for all chambers in the trial, T (T is a constant value of 5 fish x 13 timepoints per trial = 65).

Control trials were paired with gradient trials and run simultaneously. It was clear early-on that the fish had a tendency to spend most of the time in either chambers one or four, the sides of the tank, in control trials. To control for tank effects such as this, a correction was used by taking the difference between mean proportional chamber use in control trials from proportional chamber use in a gradient trial.

$$\mu = \sum_{i=1}^{8} \frac{S_i}{8T}$$

Equation 2: Mean proportional control chamber use for a given population acclimation, μ, is equal to the sum of all fish observations for a given chamber, S, from trials (i) 1-8, divided by the total number of fish observations for all chambers across the 8 trials, 8T (8T is a constant = 520).

$$X_{d,p,a,i} = \lambda_{d,p,a,i} - \mu_{d,p,a}$$

Equation 3: Shows calculation of X, the value used for preference of a given population (p) acclimation (a). This value was calculated eight times, once for each trial (i) for each population acclimation. This value also depended on the chamber (d) being analyzed for preference/avoidance.

To correct using this mean control proportional chamber use, the value μ for each population acclimation was subtracted from the eight values for λ, yielding eight control corrected values for preference in terms of proportional chamber use for each population acclimation.

Both SAS and R statistical software were used to analyze the preference data that resulted from these trials. An analysis of variance (ANOVA) was used to quantify variance in salinity preference data, testing for population, acclimation, and population*acclimation effects on preference.

$$X_{d,p,a,i} = \alpha_p \cdot p + \alpha_a \cdot a + \gamma_{p,a} \cdot p \cdot a + \varepsilon_{d,p,a,i}$$

Equation 4: Shows the calculation for the two-factor ANOVA testing for population and acclimation effects on preference, with a population*acclimation term.

Results:

To confirm that the one hour waiting period was sufficient for fish to adjust and explore the tank, fish activity was calculated over the first hour for all treatments (Figure 6). To do this, the number of fish movements was estimated using a parsimonious algorithm that calculated the minimal number of fish movements required to observe the change in fish location between 3 minute timepoints. Since the curve appears to saturate approaching 60 minutes, we are confident it was a sufficient buffer period before scoring fish for salinity preference.

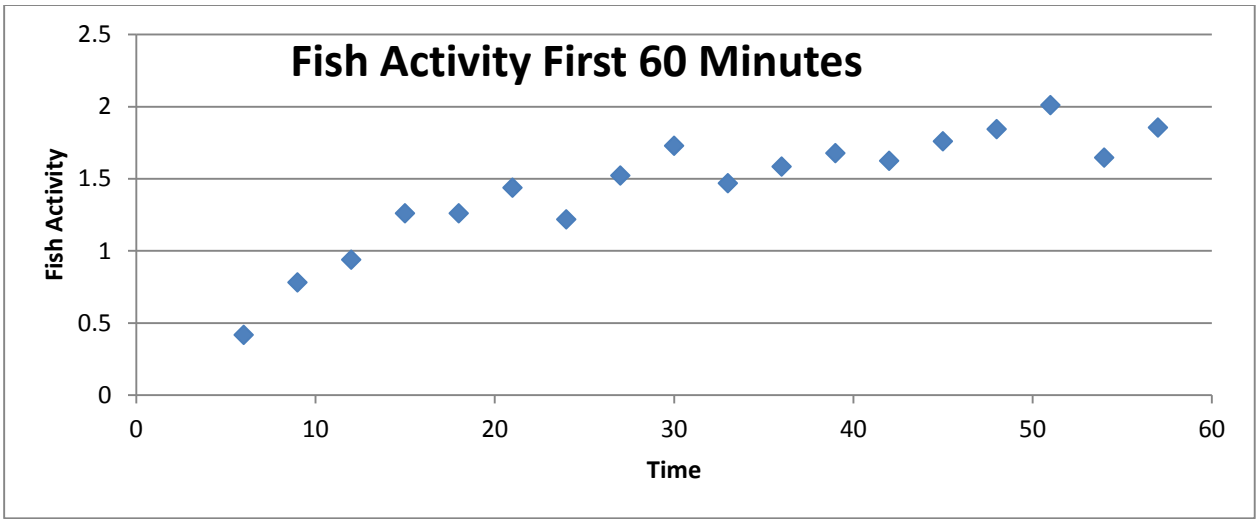


Figure 6: Fish activity was measured using a conservative calculation of fish movements between three-minute timepoints for the first 60 minutes of a trial prior to scoring for salinity preference. The curve saturates, suggesting that fish were reaching normal activity levels after the shock of transfer by 60 minutes.

The size of fish tested (Figure 7) varied by treatment group, however, there was no effect of size on preference (results not shown).

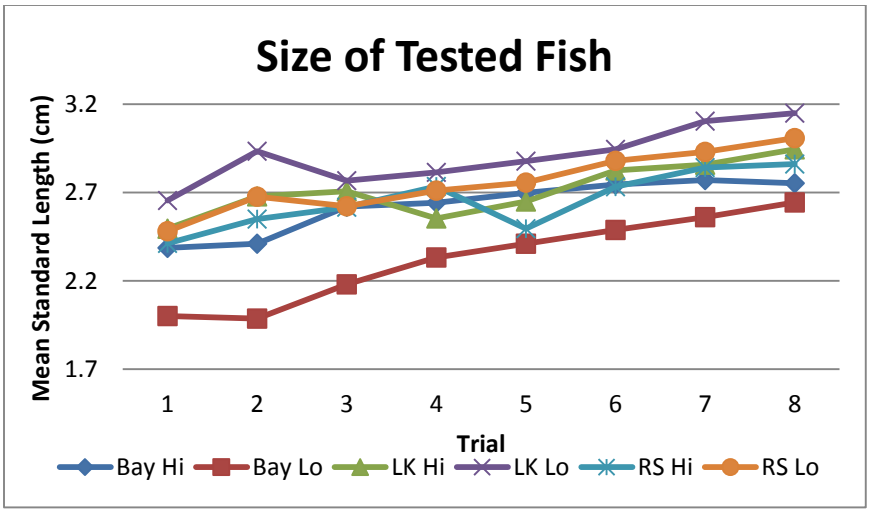


Figure 7: Mean standard length of tested fish by treatment group versus trial. Means represent mean lengths of the ten fish of each treatment group per trial (control and gradient averaged).

Pearson product-moment correlation tests (Table 4) show there is no correlation between proportional chamber use in gradient and simultaneous control trials (Figure 8), suggesting there was no trial effect. If trial to trial variability in unknown environmental factors affected fish distribution in experimental tanks (the trial effect), a correlation between simultaneous control and gradient trials would exist. Since no correlation exists, it eliminates the need for a split-plot design in which trial is nested in population, which would be the case since each trial tested only one population.

Table 4: Pearson product-moment correlations of proportional chamber use between simultaneous control and experimental tanks within a trial for each acclimation group.

	r	t	d.f.	p
Chamber 1	-0.20469	-1.4183	46	0.1629
Chamber 1*	-0.01379	-0.0915	44	0.9275
Chamber 4	-0.16871	-1.1609	46	0.2517
Chamber 4*	-0.12861	-0.87	45	0.3889

* After removal of influential values

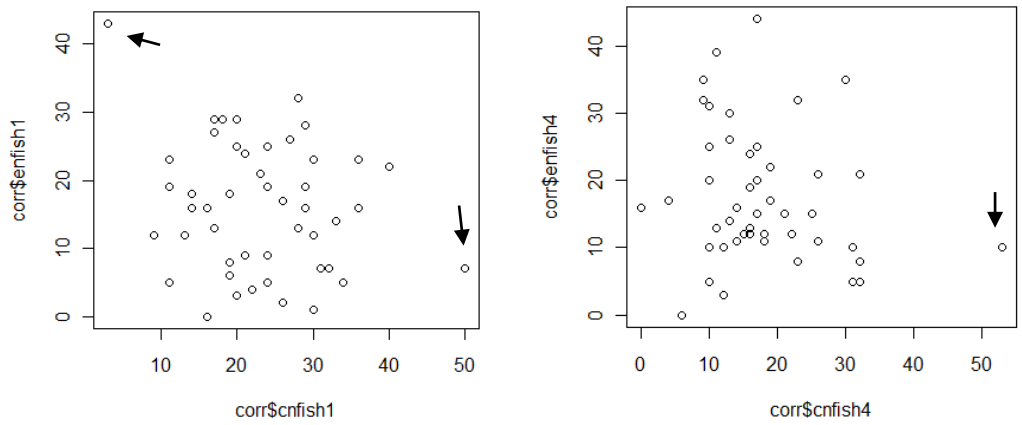


Figure 8: Correlation between gradient and control tanks per acclimation within a trial for both chamber 1 (left figure) and chamber 4 (right figure). Arrows indicate influential observations that were removed for further investigation into Pearson product-moment correlations (see Table 4).

Control tank use varied by treatment group, but generally showed an edge effect (Figure 9). Chamber 1 had slightly greater use than chamber 4 despite tank symmetry and the rotation of tanks every trial. This is because the chambers in control trials were spatially defined the same as the chambers in gradient trials. Therefore chamber 1 was the largest and chamber 4 was the smallest (see Figure 4), so one would expect in control trials that fish would, by chance, be observed more often in chamber 1 since it was defined by larger boundaries.

This edge effect, and the differences in control tank chamber size based on spatial gradient tank chamber definitions justify using a control correction, which eliminates those effects. Likewise, a control correction also eliminates a population-effect in control trial chamber use, which is apparent in Figure 9.

Mean-control chamber use per treatment group was used for these corrections rather than simultaneous control trial corrections. This was done because fish in control tanks would often migrate to one side of the tank and get "stuck" there for a large proportion of the trial. Since the tanks were symmetrical, and fish in control tanks preferred the sides, the side to which the fish

ended up being "stuck" seemed to be due to stochasticity rather than a preference for one side over the other in a tank. For this reason, and since the variation in environmental conditions from trial to trial was insignificant (Table 4), we decided to correct each gradient trial with the mean control proportional chamber use over all trials for a given population acclimation. If we used the simultaneous corrections the resulting preference values would undoubtedly be untrue since they would be marked by extremely high intertrial variability within treatment group, which would need to be alleviated via large sample sizes (the sample size of this study was only 8 trials per treatment group).

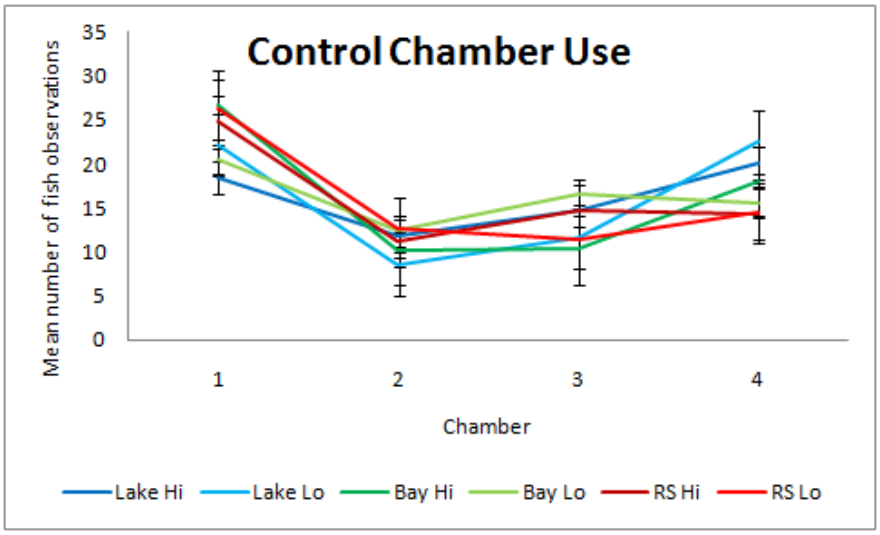


Figure 9: Edge effect in control chamber use slight variation in use between treatment groups. These control values were used to correct gradient chamber use thereby eliminating treatment differences in control chamber use and the edge effect.

We checked for normality of mean-control corrected proportional chamber use values before attempting to apply them to a statistical model as the response variable. Normal probability plots, which should be straight if data are normally distributed, as well as Shapiro-Wilk normality tests, which test the null hypothesis that data are normally distributed, both suggest these values were very marginally normal for both chamber 1 ($W = 0.954$, $p = 0.05781$) and chamber 4 ($W = 0.9323$, $p = 0.00747$). Outliers causing nonnormal distribution did not show

a trend of coming from certain treatment groups, locations, or dates. Despite possible issues with normality, we ran the proposed ANOVA's (Equation 4) since normality assumptions can be robust to outliers (Box et al. 1978), and since no simple alternative exists (there are no simple transformations that can be done to normalize proportional data since arcsin transformations are no longer accepted by the statistical community).

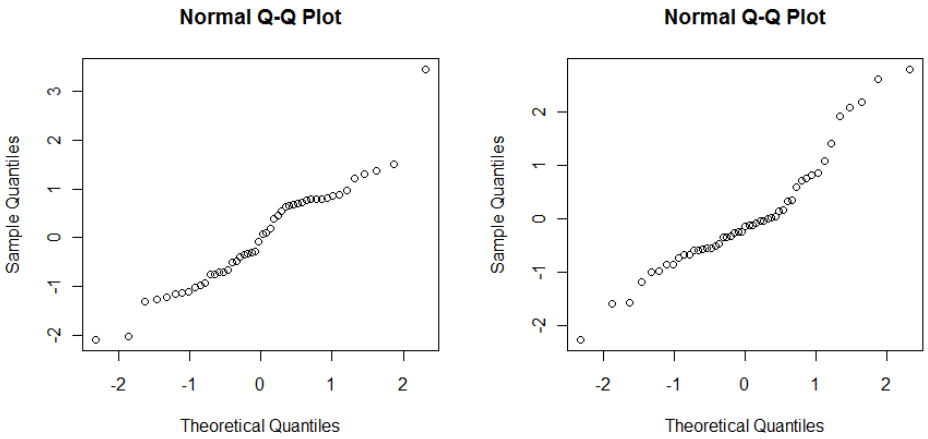


Figure 10: Normal probability plots for preference values for chamber 1 (fresh water) on the left and chamber 4 (salt water) on the right.

For the fresh water chamber (chamber 1) there were significant population ($p = 0.01730$) and acclimation ($p = 0.01488$) effects, but no significant population*acclimation interaction ($p = 0.35845$). For the salt water chamber (chamber 4) there were significant ($\alpha = 0.05$) population ($p = 0.01028$) and acclimation ($p = 0.02563$) effects, as well as a significant population*acclimation interaction ($p = 0.02117$) (See Table 5 for ANOVA results).

Table 5: ANOVA results testing population and acclimation effects on preference.

Chamber 1 (Freshwater)

	D.F.	Sum. Sq.	Mean Sq.	F	p
Population	2	0.17735	0.088674	4.4753	0.01730
Acclimation	1	0.12782	0.127816	6.4508	0.01488
Interaction	2	0.04167	0.020833	1.0514	0.35845
Residuals	43	0.83219	0.019814		

Chamber 4 (Saltwater)

	D.F.	Sum. Sq.	Mean Sq.	F	p
Population	2	0.19882	0.099408	5.1015	0.01028
Acclimation	1	0.10417	0.10417	5.3459	0.02563
Interaction	2	0.16456	0.082282	4.2226	0.02117
Residuals	43	0.83789	0.019486		

Mean preference values are reported in Table 6. RS fish preferred salt and avoided fresh water regardless of acclimation salinity.LK fish acclimated to Hi salinity avoided fresh water and those acclimated to Lo salinity avoided saltwater. Bay fish showed no preference for salt water or fresh water, but avoided both salinities at the Hi acclimation. Comparison between ancestral RS populations and derived LK populations revealed a stronger avoidance of freshwater and a stronger preference for salt water in RS fish (Figure 11). Table 7 summarizes preference/avoidance behavior for all treatments.

Table 6: Mean preference values for each population (LK, Bay, RS) and acclimation (Lo, Hi) treatment group. Positive values reflect preference while negative values reflect avoidance.

	Chamber 1, Fresh Water		**Chamber 4, Salt Water**	
	Lo	Hi	Lo	Hi
LK	-0.00769231	-0.10769231	-0.15769231	0.04807692
Bay	0.02500000	-0.15192308	-0.00769231	-0.07692308
RS	-0.17307692	-0.20576923	0.03653846	0.15769231

Table 7: Salinity preference results summary. P = preference (gradient chamber use is higher than control chamber use), A = avoidance (gradient chamber use is lower than control chamber use), and N = neutral (gradient chamber use is the same as control chamber use).

Population	**Landlocked**		**Anadromous**		**Marine**	
Treatment	LK Hi	LK Lo	RS Hi	RS Lo	Bay Hi	Bay Lo
Native Salinity (ppt)	0	0	0->35	0->35	25->35	25->35
Acclimation Salinity (ppt)	30	1	30	1	30	1
Saltwater Chamber	N	A	P	P	A	N
Freshwater Chamber	A	N	A	A	A	N

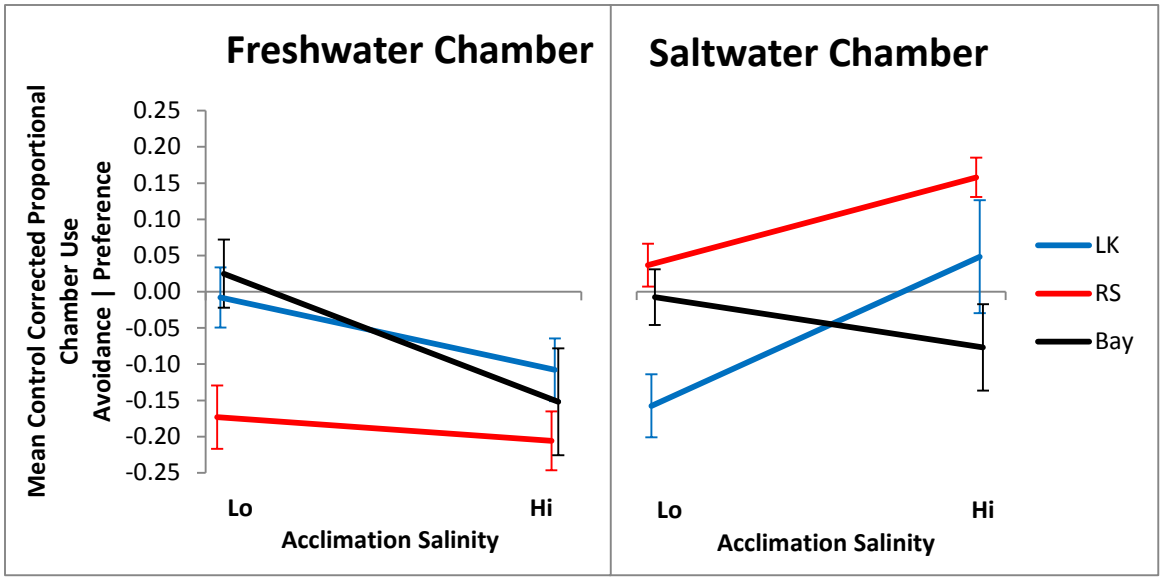


Figure 11: Comparison of preference values between landlocked LK and anadromous RS fish for the freshwater chamber (left) and the saltwater chamber (right) versus acclimation salinity. Slopes signify acclimation effects. Interpopulation differences signify evolutionary divergence. Error bars are standard error.

Since Bay treatment fish showed unclear and unintuitive trends (such as their Hi acclimation avoiding the saltwater chamber), the same ANOVA was run without the Bay data, so as only to compare between ancestral anadromous and derived landlocked populations (Table 8). This analysis brings p-values down for everything but the interactions and the acclimation effects for the freshwater chamber. Therefore, in comparison of only landlocked fish, and their nearest ancestors, we are even more confident that there are population effects, meaning that these populations have evolutionarily diverged in salinity preference behavior.

Table 8: ANOVA for effects of population and acclimation on preference, excluding Bay data.

Chamber 1 (Freshwater)

	D.F.	Sum. Sq.	Mean Sq.	F	p
Population	1	0.13882	0.13882	9.6854	0.004249
Acclimation	1	0.03521	0.035214	2.4568	0.128247
Interaction	1	0.00906	0.00906	0.6321	0.433259
Residuals	29	0.40133	0.014333		

Chamber 4

(Saltwater)

	D.F.	Sum. Sq.	Mean Sq.	F	p
Population	1	0.16074	0.16074	8.3948	0.007092
Acclimation	1	0.24	0.24	12.5342	0.001371
Interaction	1	0.00956	0.009558	0.4992	0.485511
Residuals	29	0.55529	0.019148		

Discussion:

As hypothesized, landlocked fish evolutionarily diverged from ancestral populations in salinity preference behavior. Comparison between anadromous and landlocked populations in Figure 12 elucidates trends of divergence in salinity preference.

Anadromous fish showed the strongest overall preferences. Since salinity preference is known to guide migration in anadromous fishes, it is easy to imagine that salinity preference has evolved and been selected for in anadromous populations. In the other populations, both of which experience distribution-stenohalinity, one might expect the presence of weaker preferences, as was found in this study.

Anadromous fish showed preference for salt and avoidance of fresh regardless of acclimation salinity, although the degree of preference/avoidance was affected slightly by acclimation. This result was to be expected, since the age that the fish were tested is the age at which Alaskan threespine stickleback begin their migration to sea in the wild. This result also suggests the directionality involved with seaward migration. Since these fish preferred salty water and avoided fresh water, one can imagine they would be likely to not return into fresher water at any point during migration. This behavior therefore appears to be true taxis, such that fish move in response to a salinity gradient with directionality. The evolution of this halotaxis is likely to ensure migrating fish successfully make it to sea.

In the other populations, the story is different. Landlocked fish avoided salinities they were not acclimated to. They did not prefer salinities of acclimation, however (results were neutral for salinity of acclimation, see Table 7). These fish therefore must have the ability to sense salinity, however they appear not to have a fixed salinity preference. Acclimation seems to have an affect such that fish avoid salinities they are not acclimated to. This perhaps suggests there is a behavioral barrier that would hinder landlocked fish from migrating to sea, if given the opportunity. Since they have long been acclimated to freshwaters, if landlocked fish were headed to sea due to random movements, and they experienced salt, then our results suggest they would turn around to find freshwater again, solely as a result of having freshwater acclimation. It is likely, though, that a landlocked fish could move to saltwater if other factors forced it to move into saltwater and remain there long enough for acclimation. Since in this experiment salt acclimated fish avoided freshwater, perhaps some landlocked fish would end up back in salt water given the removal of physical/geographical barriers. It is uncertain whether these populations show remnants of an ontogenetic shift in preference with age, as their ancestors have, since the study did not test shifts in preference through time. However, this temporal resolution would provide answers to many questions, and is a direction we suggest for future research.

The story of the Bay fish is less clear. Our data suggest avoidance of salt water by saltwater acclimated marine fish, and weak preferences overall. Similarly to the landlocked population, it seems intuitive that Bay fish, given their stenohaline life-history, do not have strong preference behavior since they do not migrate with respect to salinity. It therefore seems as though in fully marine stickleback populations, that a strong preference has not evolved due to the lack of pressures selecting for fish to migrate inland. However, as was mentioned in the

methods, the Bay Lo fish were from a different set of clutches than the Bay Hi fish, among some other differences. Their rearing history was different than all the other tested fish, and they were three days older. Additionally, Bay Hi fish were notably more skittish than all the other fish. We are not sure why this is, but perhaps it was a result of the location of its tank at the edge where lab workers were more often visible and close by. Since Bay trends seem to be tainted by various asterisks in rearing design and rearing tank behavior, perhaps the salinity preference behavior displayed by these fish is less reputable. The main goal of this study was to test salt preference in the landlocked population and its nearest ancestral state, however. Comparison between landlocked fish and Bay fish was therefore peripheral to the aims of this study, and our lack of confidence in Bay results caused us to stray from over-interpretation of Bay fish preference.

Comparison between the anadromous and landlocked states likely provides the best model of ancestral and derived states rather than comparison of marine and landlocked populations, since the original landlocked fish were anadromous. We see from this comparison (Figure 11) that there appears to be a shift of reaction norm towards avoidance of saltwater and preference for freshwater from ancestral to derived state. These results suggest that over the 12,000 years since landlocking, landlocked fish have evolved. The pattern of evolution suggest that fish have either lost strength of saltwater preference and freshwater avoidance likely due to relaxed selection on preference traits no longer needed.

In the past salinity preference has not been studied as a diverged trait, but has been used for the study of species migrations and distributions. This study was the first to test for divergence between related populations of threespine stickleback. Common rearing allowed us to infer evolutionary trends that have led to the discovery that salinity preference appears to be affected by relaxed selection, such that landlocked fish have a taste for fresher water, and they

have lost the strong halotaxis behaviors characteristic of ancestral anadromous populations. Our experiment used thorough control trials, often absent from earlier preference studies, which helped us to confidently eliminate tank and trial effects. Once we learn more from the ongoing studies on divergence in the osmoregulatory physiology between these populations, we will better know how closely the loss of osmoregulatory plasticity is coupled with loss of salinity preference.

We suggest that future research include a time factor, as mentioned above, to possibly observe ontogenetic shifts in salinity preference. Likewise, including a greater number of populations, and in particular, landlocked populations, could help determine if there is a trend in divergence for all landlocked populations. Simultaneous control corrections could be used in situations with a greater sample size.

We found that derived landlocked populations have evolved in salinity preference behavior after 12,000 years of landlocking by comparison with their anadromous ancestors. Landlocked fish have, to an extent, lost their preference for saltwater and their avoidance of freshwater, although these behaviors are acclimation-dependent. If the physical/geographical barriers that have prevented landlocked fish from seaward migration were removed, at this point it seems as though some behavioral barriers have evolved through relaxed selection that may hinder their successful migration. Therefore, this study provides evidence that the evolution of freshwater fish without physical/geographical barriers to seaward migration could be the result of landlocking for a significant period of time followed by removal of the physical/geographical barriers.

Funding: University of Connecticut Office of Undergraduate Research Summer Undergraduate Research Fund and University of Connecticut Department of Ecology and Evolutionary Biology Katie Bu Award Fund

Acknowledgements: Jeff Divino and Jon Velotta for constructing the tanks and helping with fish care, John Baker and lab for providing embryos, Steve Ehrlich for fish care help

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