Stony Brook University

Variation of Spatial Strategies and the Telencephalon of the Threespine Stickleback (*Gasterosteus aculeatus*) in Relation to Inferred Ecology

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Peter Jung Park

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Abstract of the Dissertation

Variation of Spatial Strategies and the Telencephalon of the Threespine Stickleback (*Gasterosteus aculeatus*) in Relation to Inferred Ecology

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The importance of habitat type for the diversification of spatial strategies and telencephalon morphology was investigated in threespine stickleback fish (*Gasterosteus aculeatus*). Field-caught samples from sea-run (ancestral) and ecologically diverse freshwater (derived) populations were studied. Freshwater habitats included shallow, structurally complex lakes with benthic-feeding stickleback, and deeper, structurally simple lakes with limnetic (planktivorous) forms.

While stickleback employ a variety of learning strategies to navigate within their environment, natural variation of spatial learning was studied because, unlike other strategies, it covaries with the size of a neuroanatomical structure. Spatial learning in relation to inferred ecology, ancestry, and experience was investigated using a T-maze. Benthic and limnetic

stickleback populations were compared, and benthics exhibited superior spatial learning compared to limnetics. In another study, a sea-run population was used to infer the ancestral condition for spatial learning in stickleback. Spatial learning was probably present in the ancestor and retained in freshwater populations as an adaptation for benthic foraging. However, lab-bred lacustrine fish performed poorly compared to their field-caught counterparts, indicating that experience is important for spatial learning.

Using field-preserved stickleback populations, I tested the hypothesis that relative size (adjusted for overall brain size) of the telencephalon is larger in benthics that occupy spatially complex habitat compared to limnetics from habitats with less structure. Contrary to expectations, field-preserved benthic populations did not consistently have larger relative telencephalon sizes than limnetic populations. However, the telencephalon of field-preserved sea-run and benthic populations was more convex laterally than that of limnetics. Although relative telencephalon size was not always larger in benthics compared to limnetics, convex telencephalon shape may indicate enlargement of the dorsolateral region, which is homologous to the tetrapod hippocampus, and greater relative size of the hippocampus is associated with superior spatial learning.

Building on these results, the importance of genetic and environmental factors to telencephalon morphology was studied. Field-preserved, lab-held (i.e., field-caught fish held in aquaria for 90 days), and lab-bred fish from benthic, limnetic, and anadromous populations were compared. An ecotypic pattern for telencephalon shape differences that was similar to previous results was detected in field-preserved and lab-held fish, but these differences disappeared in lab-bred fish. Relative telencephalon sizes of field-preserved fish were larger than those of lab-held and lab-bred ones. Taken together, these results suggest that experience, like spatial learning, is

important to telencephalon morphology. Thus, freshwater threespine stickleback appear to possess considerable telencephalon plasticity that may have been retained since the ancestor.

Dedication

This dissertation is dedicated to my parents, Chan Soo and Yoon Ja Park, and my brother, Philip Jung Park, for their unconditional love, support, and strength.

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Chapter 1

Ecologically Divergent Populations of Threespine Stickleback (*Gasterosteus aculeatus*) Utilize Multiple Learning Strategies Based on Visual Information

ABSTRACT

Teleost fishes can use visual information to learn landmarks, an algorithm, or a spatial representation of their surroundings to navigate within an environment. The existence of countless independently derived freshwater populations of threespine stickleback (*Gasterosteus aculeatus*) makes it an excellent model to study the relationship of these learning strategies to environmental differences. Stickleback populations with benthic or relatively planktivorous diets from Cook Inlet, Alaska were used to explore the potential ecological diversification of these learning strategies (Experiment 1) and possible confounding effects of non-visual information during maze learning (Experiment 2). In Experiment 1, all three strategies were identified in both ecotypes but no one strategy was favored during probe trials. These results are consistent with previous findings for landmark and algorithmic strategies using sympatric benthic and limnetic stickleback from British Columbia. However, interpretation of Experiment 1 is complicated and could have been influenced by natural and statistical properties of the samples. Experiment 2 revealed that the use of odor or sound cannot adequately explain the results from Experiment 1, supporting the conclusion that *G. aculeatus* uses primarily visual information during maze learning. Individual *G. aculeatus* from Cook Inlet, AK exhibit multiple learning strategies, including spatial learning, which mirror those in birds and mammals. Thus, *G. aculeatus* has excellent potential as an evolutionary model for the behavioral ecology of spatial learning.

INTRODUCTION

As in birds and mammals (Sherry, 1998), teleost fishes can use several visual learning strategies during foraging. Teleosts can learn the location of a food reward by associating it with a conspicuous visual landmark ("cue learning", *sensu* Salas *et al.*, 1996a) or by applying a turning algorithm ("turn discrimination", *sensu* López *et al.*, 2000). In contrast, spatial learning employs the arrangement of multiple and redundant visual cues (López *et al.,* 1999). While cue learning depends only on nearby (local) landmarks (see Warburton, 1990; Girvan & Braithwaite, 1998), spatial learning employs both local and distant (global) landmarks (see Rodríguez *et al*. 1994; Salas *et al.* 1996a, b). An individual fish is likely to possess multiple learning strategies and may alternate between them under different conditions (Hughes & Blight, 1999; Odling-Smee & Braithwaite, 2003a, b). This study explored the potential ecological diversification of visual learning strategies using a well-studied evolutionary model fish species, the threespine stickleback (*Gasterosteus aculeatus* Linnaeaus).

The biology of *G. aculeatus* has been studied extensively (Wootton, 1976; Bell & Foster, 1994; Paepke, 1996; Östlund-Nilsson *et al.,* 2007). It is primitively a marine or sea-run (anadromous) species but has repeatedly colonized and adapted to diverse freshwater habitats (Bell, 1976, 1995). Adaptation of derived lake populations for different foraging demands has resulted in predictable ecological and phenotypic divergence among numerous *G. aculeatus* populations in Europe and North America. Benthic (bottom feeding) and limnetic (open-water planktivore) specialist *G. aculeatus* populations represent extremes along a dietary continuum (McPhail 1984, 1994). Benthic *G. aculeatus* prey on invertebrates on highly structured, shallow, lake bottoms, and limnetic populations feed on plankton above deeper, open waters. Benthics and limnetics are highly divergent for foraging morphology and behavior (Schluter & McPhail,

1992). Compared to benthics, limnetics have longer, narrower snouts, more and longer gill rakers (McPhail, 1984, 1992,1994), more teeth (Caldecutt *et al*., 2001), greater armor (Vamosi, 2002), a more slender body (Lavin & McPhail, 1985, 1986; Walker, 1997; Aguirre, 2007, 2009) and head form (Willacker *et al.*, 2010), and a more triangular dorsolateral region of the telencephalon (Park & Bell, 2010). Compared to benthics, limnetics exhibit more conspicuous courtship behavior (Foster, 1994), less male-male aggression (Scotti & Foster, 2007), and do not cannibalize young defended by conspecific males (Foster, 1994). Limnetics are also better at handling plankton (Bentzen & McPhail, 1984). However, benthics can remember how to handle prey items longer (Mackney & Hughes, 1995) and are superior spatial learners than limnetics (Odling-Smee *et al*., 2008; Park, in preparation).

Odling-Smee *et al*. (2008) predicted that benthic *G. aculeatus,* which occupy structurally complex habitat, should favor cue learning while limnetics from open-water areas should prefer turn discrimination. Contrary to expectations, they found that sympatric benthics and limnetics from British Columbia did not differ in their use of these two strategies. However, benthics learned the maze sooner than limnetics, and while this result could reflect superior spatial learning ability by benthics (Odling-Smee *et al*. 2008), spatial learning was not directly measured in the study. Thus, the present work complements Odling-Smee *et al*. (2008) by incorporating spatial learning and determining whether it is ever used more than cue learning and turn discrimination. In Experiment 1 of the present study, the possibility of cue learning, turn discrimination, and spatial learning was explored using allopatric freshwater populations of *G. aculeatus* from Alaskan lake populations. While ecological and morphological differences between allopatric benthic and limnetic ecotypes are not as divergent as those of sympatric benthic-limnetic species pairs (Schluter & McPhail, 1992), sympatric benthic-limnetic species

pairs have not been observed in Alaska (M.A. Bell & S.A. Foster, personal communication). Therefore, differences among derived, allopatric, freshwater populations are a better representation of behavioral diversity in *G. aculeatus* as a whole.

Three benthic and three limnetic populations were selected for Experiment 1. Like Odling-Smee *et al*. (2008), cue learning and spatial learning was expected to be associated with living in structurally complex habitat, while turn discrimination may be associated with the less complex habitat of limnetics. In Experiment 2, the potential confounding effects of odor or sound on learning in the T-maze was tested in one benthic and one limnetic population. The use of non-visual information was expected to be negligible relative to learning based on visual cues because stickleback populations were unable to track olfactory cues within a T-maze (see Odling-Smee & Braithwaite, 2003b).

MATERIALS AND METHODS

Field Samples

In June 2005, field-caught samples from Corcoran (61.574°N, 149.688°W), Mud (61.563°N, 148.949°W), and Willow (61.444°N, 150.033°W) lakes, which contain benthic *G. aculeatus*, and from Long (61.578°N, 149.764°W), Lynda (61.571°N 149.835°W), and Nancy (61.685°N, 150.000°W) lakes, which contain limnetic populations, were captured to study ecotypic divergence of visual learning strategies (Experiment 1). In June 2008, field-caught samples from Corcoran and Long lakes were collected to test for confounding effects of odor or sound during maze learning (Experiment 2). Park and Bell (2010) present information on dietary, phenotypic, geographic, and ecological properties for all but the Nancy Lake population. The relative littoral area, a measure of euphotic zone depth in a lake with lower values indicating less

littoral habitat, for Nancy Lake is 20.891, which is lower than those of Lynne and Long lakes (M.A. Bell unpublished data), and Nancy Lake fish have slender, limnetic body shapes (Aguirre 2007).

Specimens of *G.aculeatus* were collected using 3.18 mm mesh unbaited Gee minnow traps set overnight (ca. 24 h) in heterogeneous microhabitats submerged in < 1 m depth and < 3 m from shore. Due to high mortality of senescing field-caught adults in captivity, only pre-reproductive, one-year old fish were used. Ambient water temperature in lakes was between 15° and 20° C. Live specimens were transported to the University of Alaska Anchorage and kept in outdoor pools in aged tap water for 24-48 hours and prepared for shipping. They were placed in plastic bottles that were nearly full of water and aerated while they were cooled to 5-8° C. The volume of water in the bottles was reduced to about one-third of their total volume, sealed, placed into an ice chest with freezer packs, and shipped overnight to Stony Brook University. Upon arrival, the fish were thermally acclimated over several hours and then transferred to 60 l aquaria maintained at 18°C and 3 ppt artificial seawater (i.e., Instant Ocean sea salt, Aquarium Systems Inc.). The aquaria were filtered continuously with a sponge filter (i.e., Hydro-Sponge II, Aquarium Technology Inc.) and hanging power filter (i.e., Aquaclear 30 power filter, Rolf C Hagen Corp.). The fish were fed thawed frozen adult brine shrimp daily. As a standard procedure to optimize the health of field-caught fish, all fish were treated with an antibiotic (i.e., Nitrofurazone, Aquatrol Inc. Pharmaceutical Division) and parasiticide (i.e., PraziPro™, Aquascience Research Group Inc.) after fourteen days in captivity. Experiments with these fish commenced four weeks after their arrival to Stony Brook University. Acquisition and treatment of animals used in this study were approved by the Alaska Department of Fish and Game and by

the Institutional Animal Care and Use Committee (IACUC) at both Stony Brook University and the University of Alaska Anchorage.

Maze Apparatus

A four-arm maze was constructed from 1 cm thick black plexiglass (Fig. 1). Each arm was 30 cm long, 10 cm wide, and 20 cm high. Vertical grooves cut along the height of each arm near their intersection allowed any arm to be closed off using a 3 mm thick, black plexiglass wall to produce the T-maze. Two of three remaining arms that were located 180° apart in the T-maze had a 3 mm-thick black plexiglass wall with 51 x 25 mm cut-out doors in the center. Each of these walls was 15 cm away from the end of the arm, creating a room in which food could be placed. A sliding trap wall located 15 cm away from the end of the third arm created the starting area. This trap wall was lifted by the observer using a clear, monofilament thread at the beginning of each experimental trial. The maze was submerged in a 100 cm diameter x 30 cm high circular pool filled with 3 ppt artificial seawater to a depth of 19 cm.

Pre-Training

For both experiments, pre-training trials were administered to familiarize the fish with experimental conditions. During pre-training trials, all four arms were accessible and no walls with doors were used. Thawed frozen adult brine shrimp were placed in a plastic culture dish at the end of each of all four arms. Fish were motivated by food restriction for 24-36 h prior to pre-training trials and fed only in the maze. The fish from each population were divided into two sets of similar sample size. Each set of fish from a population was allowed to swim together freely

for one hour during pre-training trials. One pre-training trial for each set was administered every other day for up to three trials.

After the final pre-training trial, each fish was placed individually in a 13 cm x 8 cm x 26 cm (0.5 cm thick) plexi-glass holding compartment to keep track of it during an experiment. Twelve of these compartments were suspended side-by-side in up to eight sixty-liter aquaria with 3 ppt artificial saltwater filtered continuously with a sponge filter (i.e., Hydro-Sponge II, Aquarium Technology, Inc.) and hanging power filter (i.e., Aquaclear 30 power filter, Rolf C Hagen Corp.). A circular hole (radius = 37 cm) covered with 1 mm-mesh located on either 13 cm x 8 cm side of a compartment allowed the filtered water to pass constantly through all the compartments in an aquarium. Each fish was housed in the same compartment during an experiment, and the compartments were randomly rearranged among the aquaria on a daily basis to eliminate potential confounding effects of interactions with neighbors and water quality differences across aquaria. All aquaria were maintained on a 12 hour light: 12 hour dark photoperiod at 18°C.

Experiment 1: Multiple Visual Learning Strategies

Experimental Enclosure

A U-shaped enclosure was constructed to minimize visual disturbances from outside the maze. Three 180 cm x 180 cm white curtains were connected end-to-end and surrounded the maze and pool (Fig. 1a). The enclosure had a height of 150 cm and maximum length of 250 cm. The open side was 180 cm wide, but a 50 cm tall solid shelf, 61 cm screen television, videocassette recorder, and camera stand obstructed visual disturbances at this end of the enclosure. Four fluorescent light bulbs (i.e., Sylvania Gro-Lux, 40W) within a ceiling fixture

(i.e., Lights of America, Model No. 8045: 118V 60Hz 70W 8A) illuminated the enclosure from directly overhead to minimize lighting differences among the maze arms. The video camera lens was 120 cm above the top of the maze. The enclosure was accessible through a slit between overlapping curtains near the opposite end from the camera stand.

Training

Three training trials were administered to each subject every second day. The starting area of the T-maze was located directly under the video camera. Before each trial, four thawed, frozen, adult brine shrimp were placed in a plastic culture dish in one of the two rooms. The fish sample from each lake was divided into two sets to test for a directional bias in the maze. The fish in one set were given their food reward in the room to the left of the starting area during the experiment (i.e., left-assigned fish), while fish from the other set were fed to the right (i.e., right-assigned fish). Subjects were motivated by food restriction for 24-36 h between training days. The order of subjects run in the maze was randomized among trials using a computerized random number generator.

To encourage cue learning during training, a conspicuous plastic plant was always positioned to the left of the door into the food-reward room. Cumulative trials to reach the criterion of nine out of ten (Phase I, see below) or four out of five (Phases II and III) correct trials (Fig. 2) were recorded. At the beginning of each trial, the subject was placed in the starting area at the base of the maze for 60 s after which the trap wall was raised remotely by the observer. A trial was completed when the fish entered the food-reward room and fed, after which it was netted and returned to its holding compartment. If the subject did not solve the maze within 15 min after the trap wall was raised, it was "encouraged" by gently prodding it with an 8

cm x 7 cm aquarium net to enter the food-reward room. Encouragement is a standard method used when fishes cannot solve a trial (see Salas *et al.* 1996a), and it is intended to feed them and to give them the same learning experience as the fish that found the reward on their own. All encouraged fish were observed feeding in the food-reward room before being transferred to their holding compartment. To eliminate odor cues, half of the water in the maze was replaced with water from the pool after every subject was run, and a complete water change for the pool and maze was performed after each trial. The maximum number of trials allowed for an experimental phase was 45, which applied only to Phase 1 because subjects that achieved the first criterion did so for all others or died before completion of the phase.

Probe Trials

Three training phases, each of which ended with a different probe trial, were administered to distinguish among learning strategies (Fig. 2). Probe trials were used to single out one learning strategy to the exclusion of others: Phase I for cue learning, Phase II for spatial learning, and Phase III for either spatial learning or turn algorithm. During a probe trial, a food reward was placed in both rooms so that subjects were not penalized for their room choice. Only the subject's first room choice was recorded, and the fish was removed before it had a chance to enter the other room.

The Phase I probe trial identified cue learning to the exclusion of other strategies. A criterion of nine out of ten consecutive trials without a wrong-room error was used to mark the completion of Phase I. During the Phase I probe trial, the plastic plant was relocated to the left-side of the door outside the untrained room. Subjects that chose this room must have learned to

associate the plant with the food reward thereby using cue learning, but subjects that chose the other room could have used turn discrimination, spatial learning, or both to find the food.

During Phase II, fish were re-trained under the same conditions used during Phase I training. A criterion of four out of five correct choices was used to indicate re-learning the maze. The Phase II probe trial was used to detect spatial learning to the exclusion of other strategies. This was achieved by re-orientating the T-maze by 180° such that the spatial relationship between the starting area and distant extra-maze cues (i.e., television, videocassette recorder, lights, video camera and stand) was inverted, while the conspicuous local intra-maze cue (i.e., plastic plant) remained unchanged in relation to the reward location since the training trials. Subjects that chose the room with the plant could have used either the plastic plant (i.e., cue learning), turn discrimination, or both. In contrast, fish that entered the opposite room must have used spatial learning because the location of this room during training trials was the same relative to only extra-maze cues.

During Phase III, the pool and T-maze were set back to the original training orientation of Phase I, and training trials were resumed. Four out of five trials without a wrong-room error was used again as the criterion for re-learning the maze. For the Phase III probe trial, the T-maze was again re-orientated by 180°, but the plastic plant was removed to determine whether spatial learning or turn discrimination was used in the absence of a conspicuous intra-maze cue. Previous work (Hughes & Blight, 1999) indicated that, in some fish, turn discrimination is preferred when conspicuous visual cues had been removed.

Experiment 2: Influence of Odor or Sound

Experimental Enclosure

A 92 cm long, 78 cm wide, and 131 cm high enclosure lined with opaque white shower curtains was constructed to minimize disturbances occurring around the maze and to eliminate any conspicuous distant extra-maze cues (Fig. 1b). A fluorescent light fixture (i.e., Lights of America, Model No. 8045: 118V 60Hz 70W 8A) with one bulb (i.e., Sylvania Gro-Lux, 40W) was attached along each length of the enclosure. A 51 cm tall video camera stand that rested atop the enclosure was constructed from PVC, plastic, and wood. The distance from the camera lens to the top of the maze was 119 cm. Observer entry into the enclosure was through a slit between overlapping shower curtains along the middle of one width. The video camera was connected to a videocassette recorder and 61 cm screen television monitor from which an observer coded behaviors during trials.

Training

Field-caught pre-reproductive *G. aculeatus* from Corcoran and Long lakes were used to investigate the potential confounding effects of odor or sound during learning in the T-maze. Unlike Experiment 1, the location of the food reward was randomized from trial-to-trial between the two rooms that were 180° apart, except that $\leq$ 3 consecutive trials had the food in the same room. Before each trial, four thawed frozen adult brine shrimp were placed inside the food-reward room in a plastic culture dish. Two drops of liquid from the thawed brine shrimp stock were placed $\leq$ 5 cm in front of the entrance to the food-reward room to create an odor cue at this door. As in Experiment 1, subjects were motivated by food restriction between training days. Due to logistical limitations, the training phase for Experiment 2 had to be changed in two ways

relative to the previous experiment. In Experiment 2, two instead of three trials were administered every second day and the maximum number of trials was 35 instead of 45. Preliminary studies indicated that two or three trials every other day with an upper limit of 35 trials is sufficient to detect improvement in learning in the T-maze (Park, in preparation), and other investigators were able to detect learning in *G. aculeatus* using a regimen of only one trial every two days (Girvan & Braithwaite, 1998, 2000). Therefore, results between Experiments 1 and 2 were considered suitable for comparison.

An external aquarium air pump (i.e., 115 V AC, Tetra/Second Nature) without an airline tubing connection was used to generate a constant acoustic stimulus. It was positioned outside the experimental enclosure but adjacent to the food-reward room during all training trials such that the locations of the sound and food were always coupled (Fig. 2). The pump was placed outside of the enclosure to avoid the creation of surface waves near the food reward arm, which could have generated unintended visual cues in the form of lighting differences between arms. Nine out of ten correct room choices was the criterion to solve the maze. At the beginning of each trial, the subject was placed in the starting area at the base of the maze for 60 s after which the trap wall was raised remotely by the observer. A trial was considered complete when the fish entered the food-reward room and fed. If the subject did not solve the maze within 15 min after the trap wall was raised, it was "encouraged" by gently prodding it with an 8 cm x 7 cm aquarium net to enter the food-reward room. A complete water change for the pool and maze was done after all subjects completed a trial. The order in which subjects were run in the maze between trials was determined using a computerized random number generator. Probe trials were planned but could not be analyzed because too few fish solved the maze (Corcoran: N=2 of 14; Long: N=1 of 14).

Boldness and Exploratory Behavior (Trial 1)

The performance of each fish during its first trial in Experiments 1 and 2 was used to measure its boldness or tendency for exploratory behavior. During this trial, an individual fish was exposed for the first time to a starting area, moving trap wall, and a room with a door at either end of the arms that were 180° apart. Subjects were acclimated for 60 s before the trap wall was raised, after which all subjects either froze or darted within the starting area. This indicated that subjects were startled, and therefore, it was reasonable to assume that, during trial 1, subjects treated the T-maze like a novel environment. These startle behaviors diminished drastically over the next few trials and did not occur in later trials.

Bolder individuals will risk potential danger in return for foraging gain (Ward *et al*., 2004). They are also more likely to explore novel surroundings (see Budaev, 1997), potentially biasing them to learn about their surroundings sooner than shy (i.e., low boldness) individuals (see Burns & Rodd, 2008). Freezing is a standard behavioral measure used to determine boldness in fishes (Walsh & Cummins, 1976; Budaev, 1997; Burns, 2008) and is used by stickleback to evade detection by predators (Wootton, 1984; Huntingford & Coyle, 2007). Thus, boldness was measured as the residual of the number of total freezes regressed on the time interval during which a fish's caudal peduncle initially left the starting area and entered a room in trial 1.

Ambulation in a novel environment is a reliable indicator of exploratory behavior (Walsh & Cummins, 1976; Gervai & Csányi, 1985; Budeav, 1997; Burns, 2008), and it is typically measured as the time spent in motion (see Budeav, 1997, Burns, 2008) or the number of times a subject crosses regular intervals (Gervai & Csányi, 1985). Exploratory behavior was recorded in the current study as the number of entries that a fish made into either arm or back into the

starting area during the time that a fish's caudal peduncle first left the starting area (marking the beginning of a trial) and entered a room (marking the first entry into a room).

Statistical Analyses

Biomstat version 3.30q (see Sokal & Rohlf, 1995) or STATISTICA version 9.1 was used to test for differences in learning strategies and other performance measures. The number of trials to reach each criterion was analyzed using a test that compares waiting time (or time-to-event) distributions (e.g., Gehan's generalized multi-sample Wilcoxon test, Peto and Peto's Wilcoxon two-sample test, see Lee & Wang, 2003) across samples. In Experiment 1, subjects that could not achieve the Phase I criterion after 45 trials were assigned the maximal score of 45 trials for Phase I. Attrition occurred during Phases II and III of Experiment 1, but these tests can incorporate progressively censored (e.g., missing data for subjects that did not survive) as well as singly censored data (e.g., missing data for subjects that survived but did not achieve a criterion) (see Lee & Wang, 2003).

Differences in learning strategies used during probe trials were analyzed by a G-test for independence. If a fish was lost to attrition, it was excluded from the analysis of the following probe trial. Because the Phase III probe trial had the smallest sample sizes compared to other probe trials, a multinomial exact test was used because in addition to giving exact probability values, it is statistically more powerful than the G-test when the expected frequency is less than 10 for a sample (Conahan, 1970), which was the case for limnetics.

In Experiment 2, the waiting time distributions for trials to reach criterion were compared using the same tests as in Experiment 1. In addition, a multinomial exact test was used to compare the proportion of fish that solved the maze in Experiment 2 with those from the same

population in Experiment 1; the expected values were the proportions of fish that solved the maze in Experiment 1.

The confounding variables boldness and exploratory behavior were analyzed using ANCOVA and ANOVA, respectively. All subjects were used for these tests because every fish participated in the first trial. Time interval data were $\log_{10}$-transformed, and count data (i.e., number of freezes, ambulation) were square-root transformed.

RESULTS

Experiment 1

This experiment investigated differences in learning rate and propensities for different learning strategies between ecologically contrasting lake populations (Table I). A Gehan's generalized multi-sample Wilcoxon test failed to detect differences in the waiting time for trials to reach the Phase I criterion (i.e., nine out of ten correct trials) among benthic (χ_2^2= 0.034, p=0.98) and among limnetic (χ_2^2= 4.547, p=0.10) samples, and therefore, samples within ecotype were pooled. A Peto and Peto's Wilcoxon test (Peto & Peto, 1972) failed to detect differences between benthic and limnetic waiting time distributions for trials to reach the criterion for Phase I (W=0.590, p=0.56; Fig. 3). Similarly, trials to reach the Phase II criterion (i.e., four out of five correct trials) did not differ among benthic (χ_2^2=0.506, p=0.78) and limnetic (χ_2^2=-1.582, p=0.11) samples. Upon pooling samples within ecotype, there was no significant difference between benthics and limnetics for trials to reach Phase II (W=1.337, p=0.18; Fig. 3). Finally, trials to reach the Phase III criterion (i.e., four out of five correct trials) again did not differ among benthic (χ_2^2= 2.023, p=0.36) or among limnetic (χ_2^2=3.183, p=0.20) samples. After samples within ecotypes were pooled, no ecotypic differences were detected for trials to reach Phase III

(W=-1.663, p=0.10; Fig. 3). Thus, benthics and limnetics did not differ in the number of trials needed to reach each criterion.

Differences in learning strategies were analyzed using a G-test on probe trials. The room that a subject entered during a probe trial was recorded, and the ecotype group was tested for deviation from the 50 : 50 expectation, which would indicate no propensity for a strategy. The Phase I probe trial was designed to detect cue learning. The plastic plant was re-located to the opposite door. Fish that qualified for this probe trial presumably learned the maze with a propensity for a particular learning strategy, and fish that chose this untrained room with the plant must have been using cue learning while those that chose the other room used turn discrimination, spatial learning, or both. During the Phase I probe trial, 76% of benthics (N=41, G=5.642, $p<0.05$) chose the bare trained door more often than the untrained room with the plant, indicating a propensity to use turn discrimination and/or spatial learning. Seventy per cent of limnetics also preferred this room but this result did not differ significantly from 50 : 50 (N=27, G=1.529, p=0.13).

The Phase II probe trial tested for turn discrimination to the exclusion of cue learning and spatial learning by rotating the T-maze by 180° without changing other aspects of the maze. Thus, fish that chose the room with the plastic plant used cue learning, spatial learning, or both while those that selected the unmarked room used turn discrimination. Seventy per cent of the benthics (N=40, G=3.291, p=0.07) and 52% of the limnetics (N=21, G=0.024, p=0.88) chose the door with the plastic plant more often, which would have implied a propensity for turn discrimination and/or cue learning, but these results were not significant.

The plastic plant cue was removed in the Phase III probe trial and the orientation of the T-maze was rotated by 180°. Removal of the plant eliminated cue learning, and thus, only turn

discrimination or spatial learning was tested. Sixty-two per cent of benthics (N=39; G-test: G=1.048, p=0.31; Multinomial Exact Test: p=0.10) and 59% of limnetics (N=17, G=0.266, p=0.61; Multinomial Exact Test: p=0.31) favored the room indicative of turn discrimination, but again, these results were not significant. Overall, benthic and limnetic fish exhibited multiple visual learning strategies. Although fish seemed to prefer the room consistent with use of turn discrimination, statistical tests indicated that there were no strategy propensities except in benthics during the Phase I probe trial. During this trial, benthics favored turn discrimination and/or spatial learning over the plastic plant cue, but benthics did not favor either strategy when the plant was removed (i.e., Phase III probe trial).

Boldness and exploratory behavior are confounding variables that could have influenced the results. To compare boldness, an ANCOVA of square-root transformed number of freezes was used with log of the time interval between initial exit of the starting area and entry into a room as the covariate. No differences were detected among limnetic ($F_{2,28}$=1.479, p=0.25) or benthic ($F_{2,44}$= 2.145, p=0.13) samples, and thus, samples were pooled within ecotype. Benthics and limnetics did not differ for boldness ($F_{1,77}$= 0.692, p=0.41). Exploratory behavior was analyzed using a three-level nested ANOVA with square-root transformed ambulation during trial 1 as the dependent variable and the assigned side within population within ecotype as the grouping variable. There was no effect of exploratory behavior for ecotype ($F_{1,4}$=0.420, p=0.55), population ($F_{4,6}$=3.109, p=0.10), or T-maze side ($F_{6,68}$=0.936, p=0.48). Thus, boldness or exploratory behavior did not differ between ecotypes.

Overall, there were no ecotypic or population differences for trials to reach criterion. There was also no general ecotypic difference for learning strategy during probe trials.

Furthermore, neither of the potential confounding factors, boldness or exploratory behavior, influenced these results.

Experiment 2

This experiment concerned the influence of odor or sound on learning in a T-maze and compared only Corcoran (benthic) and Long (limnetic) lake subjects. Very few fish from either sample solved the maze (Corcoran: N=2 of 14; Long: N=1 of 14). A Peto and Peto's Wilcoxon test did not detect a significant difference between Corcoran and Long Lake samples for trials to reach criterion (W=0.981, p=0.33).

The confounding variables boldness and exploratory behavior were analyzed. An ANCOVA of square-root transformed number of freezes was used with log of the time interval between initial exit of the starting area and entry into a room as the covariate. No differences were detected between Corcoran and Long samples ($F_{1,25}$=0.511, p=0.48). A single classification ANOVA on square-root transformed ambulation during trial 1 also failed to detect differences between samples ($F_{1,26}$=1.957, p=0.17). Thus, the Corcoran and Long Lake samples did not differ for boldness or exploratory behavior.

Each lake sample from Experiment 2 was compared to its counterpart from Experiment 1 to identify potential confounding effects of odor or sound. Corcoran and Long Lake samples from both experiments were analyzed for boldness or exploratory behavior differences to determine if comparisons across experiments were justified. An ANCOVA failed to detect boldness differences across all four samples ($F_{3,53}$=0.937, p=0.43). Similarly, a single classification ANOVA did not detect differences for exploratory behavior across the samples

($F_{3,54}$=2.461, p=0.07). Thus, samples from the same lake population could be compared between experiments.

Because the maximal number of trials allowed for Experiment 2 was 35, subjects from Experiment 1 that failed to achieve the criterion in less than 36 trials were designated the maximal 35 trials. This adjustment reduced the proportion of fish that solved the maze in Experiment 1 from fifteen to ten for Corcoran Lake and nine to eight for Long Lake. A multinomial exact test using these results from Experiment 1 as expected values indicated that fish from Experiment 1 solved the maze sooner than their counterparts from Experiment 2 (Corcoran: N=2 of 14, $p<0.001$; Long: N=1 of 14, $p<0.001$). Similarly, a Peto and Peto's Wilcoxon test showed that fish from Experiment 1 outperformed their counterparts from Experiment 2 (Corcoran: W=2.368, $p<0.05$; Long: W=2.745, $p<0.05$; Fig. 4). Therefore, based on two independent analyses, fish from Experiment 1 solved the T-maze better than those from Experiment 2.

Overall, fish from Experiment 1 outperformed their counterparts from Experiment 2, which strongly indicates that *G. aculeatus* utilize visual information and that audition and olfaction contribute an inconsequential amount of information while foraging in the T-maze. Furthermore, neither of the potential confounding factors, boldness or exploratory behavior, appeared to influence these results.

DISCUSSION

Like birds and mammals, fishes can use cue learning, turn discrimination, and spatial learning strategies to navigate in their environment (López *et al*., 1999; Hughes & Blight, 1999). Benthic and limnetic populations of Alaskan *G. aculeatus* were used to investigate the relative

importance of these strategies in relation to foraging ecotype (Experiment 1) and to address the potential confounding effects of odor or sound on learning based on visual information in the T-maze (Experiment 2). Despite clear evidence that all three visual learning strategies are used in Alaskan *G. aculeatus* populations, no one strategy was chosen more often over the others. These results are consistent with results from Odling-Smee *et al*. (2008) using sympatric benthic and limnetic *G. aculeatus* populations. Furthermore, neither olfaction nor lateral line sense accounts for the results.

Derived *G. aculeatus* lake populations sampled for this study occur in a recently deglaciated region of southcentral Alaska (Bell *et al*., 1993) that was colonized by sea-run (anadromous) ancestors within the last 15,000 years (Reger & Penny, 1996). Similarities among freshwater populations from different river systems that are divergent from the anadromous ancestor probably evolved independently (Taylor & McPhail, 1999, 2000; Aguirre, 2007). Populations with similar predation regimes were selected so that the potential effects of predator avoidance behavior on learning strategies would be more-or-less constant among populations (Huntingford, 1976; Huntingford *et al*., 1994; Portavella *et al*., 1998, 2003, 2004; Brydges *et al.*, 2008; Burns & Rodd, 2008). The populations sampled are sympatric with native predatory fishes, and predatory birds and insects are present in all boreal lakes (Reimchen, 1994).

The protocol in Experiment 1 utilized three tests, each interspersed between training trials, to distinguish among the three possible learning strategies that could have been used to solve the maze. Additional training sessions could not be employed because preliminary studies suggested that *G. aculeatus* from these populations are very susceptible to mortality under experimental conditions for durations longer than that used in Experiment 1 (Park, unpublished data).

Cue learning requires the individual to learn a conspicuous visual landmark while turn discrimination utilizes an algorithm (López *et al*., 1999). Odling-Smee *et al*. (2008) hypothesized that benthic *G. aculeatus* should rely more on visual landmarks while limnetics should favor algorithmic learning. However, they did not find any learning strategy differences between members of sympatric benthic-limnetic species pairs from British Columbia. In Experiment 1 of the present study, a greater proportion of fish from both ecotypes chose the room consistent with use of turn discrimination during Phase I, II, and III probe trials (Fig. 2). Although this result was statistically significant only for benthics during Phase I, the consistency of this pattern suggests that samples may have been too small to detect a difference in the other comparisons. However, it must be concluded based on the available data that turn discrimination is not more likely to be used than cue or spatial learning in Alaskan *G. aculeatus*. This result is consistent with Odling-Smee *et al*. (2008). Turn discrimination is associated with life in habitats where visual cues are unreliable (e.g., rivers; see Girvan & Braithwaite, 1998; Odling-Smee & Braithwaite, 2003b), but only further study using larger sample sizes can clarify the possible importance of turn discrimination to locate food by lacustrine stickleback populations.

Cue learning is associated with habitat stability in *G. aculeatus* (Girvan & Braithwaite, 1998; Odling-Smee & Braithwaite, 2003b), and in other fish species, with benthic foraging (Warburton, 1990), low predation pressure (Brown & Braithwaite, 2005), territoriality (Lamanna & Eason, 2003), and migration (Fukumori *et al*., 2010). Odling-Smee *et al*. (2008) proposed that both benthics and limnetics could use visual cues similarly to explain the lack of learning strategy differences (also see Hughes & Blight, 1999, 2000). Benthics and limnetics from Cook Inlet, AK did not use a conspicuous visual cue (i.e., the plastic plant landmark) more than other strategies. Unlike Odling-Smee *et al*. (2008), who used sympatric populations of benthics and

limnetics that usually occupy non-overlapping habitats and have extreme morphological and behavioral differences (McPhail, 1984, 1992, 1994; Schluter & McPhail, 1992; Schluter, 1996), benthics and limnetics used in the present work came from different lakes. Allopatric limnetic populations that are as extreme as sympatric limnetics from British Columbia do not occur in Cook Inlet (M.A. Bell & S.A. Foster, personal communication), but both sympatric and allopatric limnetics tend to consume benthic prey during the breeding season (Schluter and McPhail 1992). Thus, although benthics live in shallow lakes that include exclusively benthic habitat, limnetics live in deep lakes that nevertheless include substantial littoral habitat, where they can consume benthic prey, and limnetic habitat off shore. Therefore, the similar responses of Alaskan benthics and limnetics may reflect similar littoral habits, which do not necessitate exclusive use of different strategies. Like benthics, limnetics may use visual cues in littoral microhabitats to locate food patches (see Hughes & Blight, 1999, 2000; Odling-Smee *et al.*, 2008; Park & Bell, 2010) or during the breeding season, when mating occurs near shore, and males establish territories that females must locate (Ridgeway & McPhail, 1984; McPhail, 1994; Odling-Smee *et al.*, 2008; Gonzalez-Voyer *et al.*, 2009). Therefore, while it is unclear why ecotypic differences for cue learning were not found, lacustrine *G. aculeatus* may generally rely on conspicuous landmarks in their native habitat for a variety of diverse functions.

While no learning strategy propensities were detected in limnetics during Experiment 1, benthics did tend to use either turn discrimination or spatial learning to the exclusion of the plastic plant landmark during the Phase I probe trial. However, like limnetics, when the plant was removed (i.e., Phase III probe trial), benthics were as likely to use turn discrimination as they were spatial learning, and these results were not influenced by differences in boldness or

exploratory behavior. It is not immediately clear how these results compare to those of Odling-Smee *et al*. (2008), who tested only cue learning and turn discrimination.

Despite a lack of difference for cue learning or turn discrimination during probe trials, British Columbian benthic *G. aculeatus* took fewer trials to reach the criterion than sympatric limnetics (Odling-Smee *et al*., 2008). The more efficient performance of benthics was suggestive of superior spatial learning in association with life in the littoral zone, but spatial learning was not directly measured in their study. Probe trials for Phases II and III in Experiment 1 of the present study identified spatial learning in a subset of fish from both ecotypes. A striking difference between the result from Odling-Smee *et al*. (2008) and that of Experiment 1 was that Alaskan benthics did not reach the criterion for learning to find the food-reward room sooner than limnetics, which would imply that ecotypic differences in spatial learning do not exist between Alaskan benthics and limnetics. However, Park (in preparation) found that *G. aculeatus* from benthic populations used in this study were better spatial learners than those from relatively planktivorous populations. In that study, cue learning and turn discrimination could not be used to solve the maze due to the experimental design. Moreover, while Odling-Smee *et al*. (2008) controlled for distant extra-maze cues by surrounding their maze apparatus with white curtains, numerous extra-maze cues (e.g., camera stand, television, videocassette recorder) were intentionally provided in Experiment 1 to create the potential for subjects to employ spatial learning. It is uncertain whether or not benthic subjects in the current study would have responded similarly if extra-maze cues were minimized. Therefore, the benthics used in Experiment 1 may have been better at spatial learning than limnetics, but this could not be detected because the methods of the current study did not control for cue learning or turn discrimination.

Unlike other strategies, spatial learning utilizes multiple and redundant sources of spatial information (reviewed in López *et al*., 1999; Jacobs, 2003). As in other fishes, spatial learning may be associated with a variety of ecological factors in *G. aculeatus*. For instance, gobies use spatial learning to memorize the array of intertidal pools (Aronson, 1951, 1971) or burrows (Markel, 1994) to evade predators. Goldfish (*Carassius auratus*) employ spatial learning to forage (Salas *et al*., 1996b), and Mexican blind cave fish (*Astyanax fasciatus*) use it to navigate within structurally complex habitats (de Perera, 2004). In addition, residence in the intertidal zone is associated with spatial learning in the fifteenspine stickleback (*Spinachia spinachia*) and corkwing wrasse (*Crenilabrus melops*) (Hughes & Blight, 1999). The demands associated with male parental care (Gonzalez-Voyer *et al*., 2009; Odling-Smee *et al*., 2008) and maintaining a territory (see Sherry, 1998) may also favor use of spatial learning. Although it is not entirely clear how spatial learning is related to ecotypic variation in *G. aculeatus*, Experiment 1 and the results of Park (in preparation) reveal that benthic foraging may be very important for this learning strategy in *G. aculeatus*.

Experiment 2 investigated the relative contribution of odor or sound to T-maze learning. Studies investigating visual learning in the T-maze in fishes have not previously considered the potential for olfaction or lateral line sense be confounding effects (but see de Perera, 2004; Webb *et al*., 2008). In *G. aculeatus*, odor is used to recognize mates (McLennan, 2003; Rafferty & Boughman, 2006) and to develop shoaling propensities with other fish (Ward *et al*., 2004). In the Mexican blind cavefish (*A. fasciatus*), lateral line sense is the primary sensory modality used during spatial learning (de Perera, 2004). Experiment 2 reveals that *G. aculeatus* primarily use vision and not other sensory modalities to learn the T-maze. For Corcoran (benthic) and Long (limnetic) lake populations, fish from Experiment 1 outperformed those from Experiment 2,

suggesting that learning during Experiment 1 was based on visual information and not odor or sound. However, it is possible that the inability of these fish to find the food using the sound cue was due to an inappropriate experimental set up that did not provide directionality because the sound was not audible in the water or it reflected around the room before it entered the water, providing no indication of the location of the source. In contrast, the location of odor should have been unambiguous. These results are consistent with another study using *G. aculeatus*, which also identified vision as the predominant sensory modality used to learn another type of maze (see Girvan & Braithwaite, 1998). Probe trials were planned for Experiment 2 to determine which modality was most important, but too few fish solved the maze (Corcoran: N=2 of 14; Long: N=1 of 14). Therefore, no tests could be performed for probe trials.

In conclusion, *G. aculeatus* from Cook Inlet, AK can utilize a variety of visual learning strategies. Since no ecotypic pattern was detected for trials to reach the criterion or for a single learning strategy, individual fish from all sampled populations seem to possess a robust repertoire of learning strategies. Contrary to expectation, benthics did not use cue learning or spatial learning to the exclusion of turn discrimination, but they did use either turn discrimination or spatial learning instead of a visual landmark. However, learning propensities disappeared when either turn discrimination or spatial learning was singled out. No learning propensities were found in limnetics. In general, these results are consistent with those of Odling-Smee *et al*. (2008) that benthics and limnetics did not show cue learning or turn discrimination differences. Experiment 1 clearly demonstrated that spatial learning exists in Alaskan *G. aculeatus* populations, which might be the case for *G. aculeatus* populations elsewhere. Spatial learning is associated with size variation of the hippocampus of birds and mammals (Healy *et al.*, 2005) and of the dorsolateral region in the telencephalon of fishes (Salas *et al*., 1996b; Vargas *et al.*, 2000;

Rodríguez *et al*., 2002), and it is possibly important in the *G. aculeatus* populations included in this study (Park & Bell, 2009). In contrast, cue learning and turn discrimination are not associated with morphological variation in the brain (see Salas *et al*., 1996b; López *et al*., 1999). The proximate mechanisms of spatial learning have been studied extensively in terrestrial vertebrates (Healy *et al*., 2005; Salas *et al*., 1996b), but exciting and truly novel discoveries may be revealed using fishes like *G. aculeatus*. Unlike other vertebrate models, the ancestral condition for any trait is observable in extant, anadromous populations of *G. aculeatus*, and its genomics has also emerged as a powerful model to understand the evolution of complex traits (Gibson, 2005; Cresko *et al*., 2007; Kingsley & Peichel, 2007). Therefore, *G. aculeatus* provides an exciting evolutionary model system to study cognition and its neuroanatomical and genetic causes. Future work should aim to identify the multiple components of learning (e.g., motivation, attention, reinforcement, discernment, memory) that may be affected during evolution of spatial learning (see West-Eberhard, 2003; Brydges *et al*., 2008; Burns & Rodd, 2008), constraints among them, and their relationship to the brain (see Warburton, 2003).

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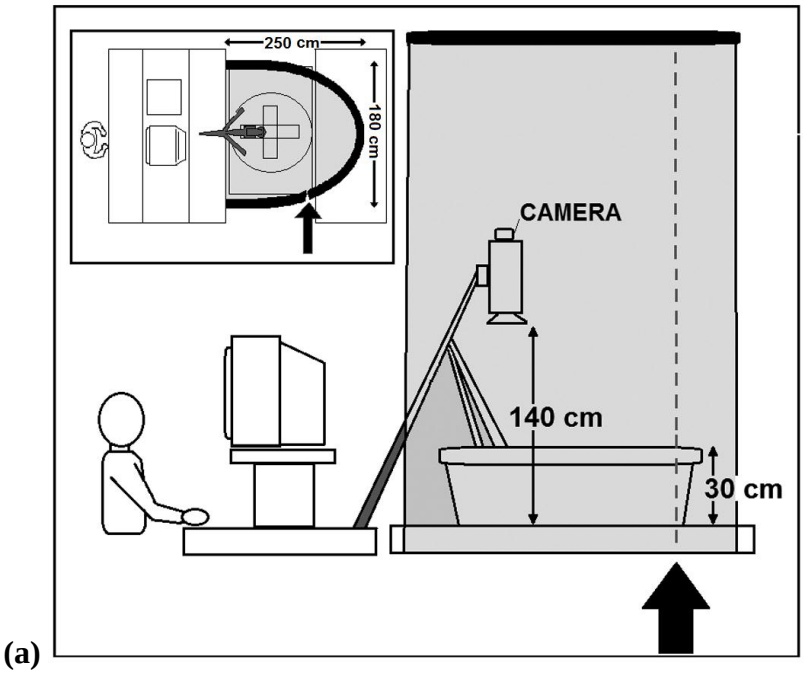
Table 1. Experimental results. The experiment number and year are shown on the left above the names and sample sizes (n) used in the experiment. See the text for methods and criteria to accomplish each experimental phase. Samples included fish that solved the maze (S), did not solve the maze (U), or were lost to attrition (A).

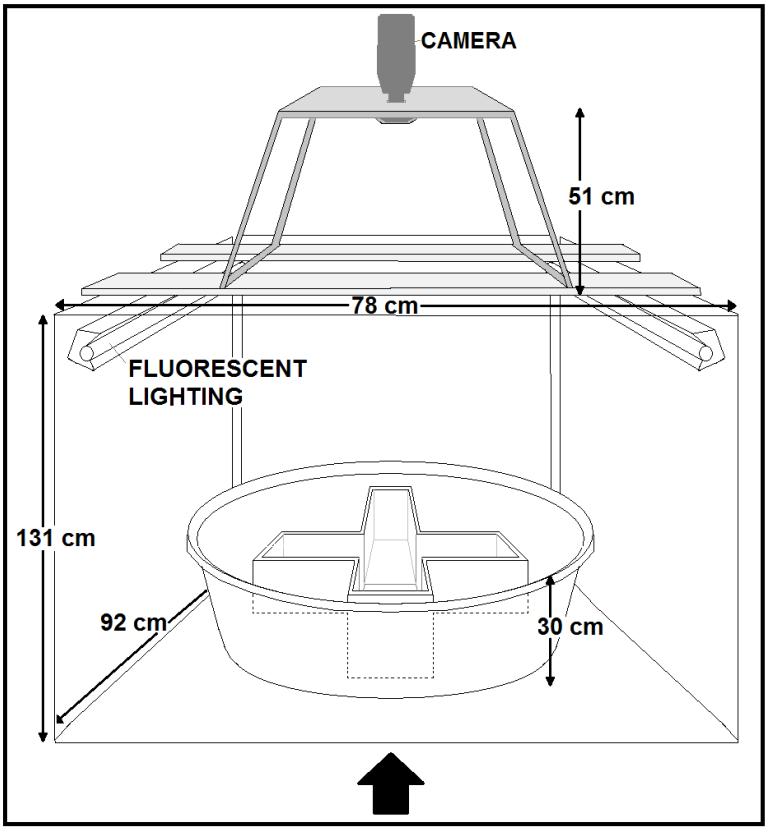
Expt/Sample	**Ecotype**	**Phase I**			**Phase 2**		**Phase 3**	
		S	**U**	***A***	**S**	***A***	**S**	***A***
Experiment 1 (2005):								
Long (n=14)*	Limnetic	**9**	5	*0*	**9**	*0*	**7**	*2*
Lynda (n=14)	Limnetic	**12**	2	*0*	**11**	*1*	**8**	*3*
Nancy (n=7)	Limnetic	**7**	0	*0*	**4**	*3*	**2**	*2*
Corcoran (n=16)*	Benthic	**15**	1	*0*	**15**	*0*	**14**	*1*
Willow (n=16)	Benthic	**14**	2	*0*	**14**	*0*	**13**	*1*
Mud (n=16)	Benthic	**12**	4	*0*	**12**	*0*	**12**	*0*
Experiment 2 (2008):								
Long (n=14)*	Limnetic	**1**	13	*0*	-	-	-	-
Corcoran (n=14)*	Benthic	**2**	12	*0*	-	-	-	-

* Results that were compared between Experiments 1 and 2.

Figure 1. Enclosures for experiments (a) 1 (plan view inset) and (b) 2. The arrows indicate the entry point for the observer into the enclosure. Drawings not to scale. See text for dimensions of four-arm maze.



(a)



(b)

Figure 2. Schematic outline of experiments. (a) Experiment 1. The top diagram indicates the training arrangement for a fish located in the starting area and trained to receive food on the right arm of the T-maze. The lower diagrams indicate the three tests used to identify the three possible learning strategies. The inferred learning strategy used to solve each test is indicated under the corresponding rooms. (b) Experiment 2. The location of the food reward was randomized trial-by-trial between arms that were 180° apart, and noise was generated by an air pump (♪) that was coupled to the location of the food.

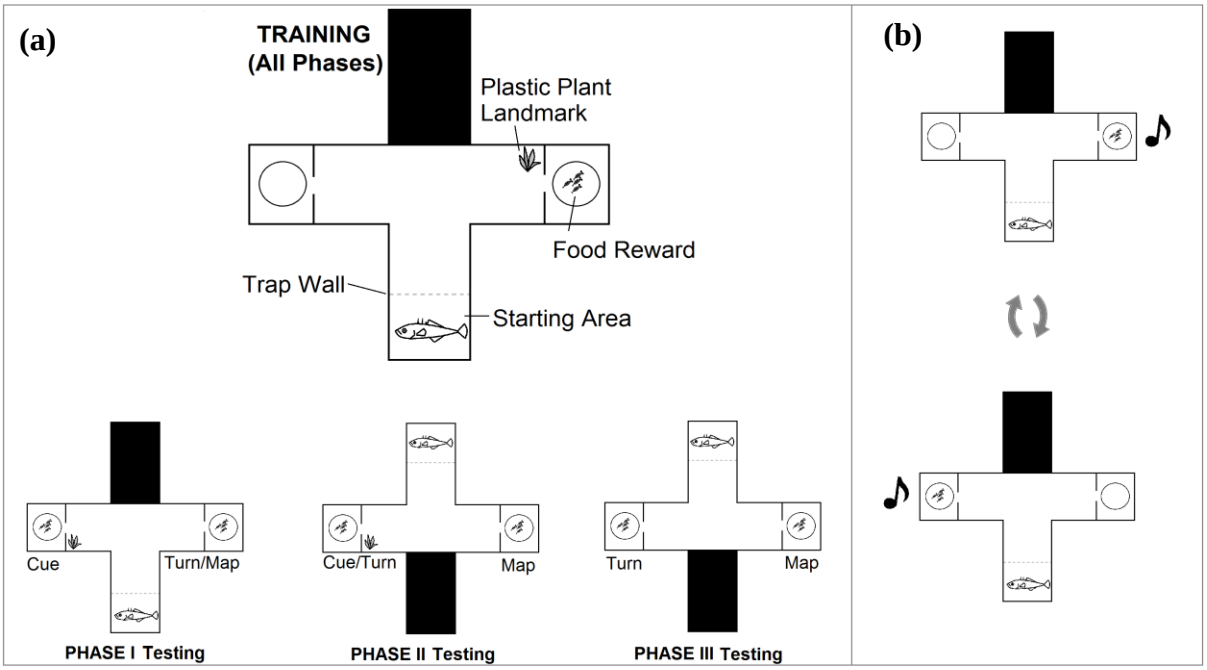


Figure 3. Mean number and standard errors of trials to reach criteria (Experiment 1). The criterion was nine out of ten (Phase I) or four out of five trials (Phases II and III) with correct room choices. Results for fish that were lost to attrition before achieving a criterion are excluded.

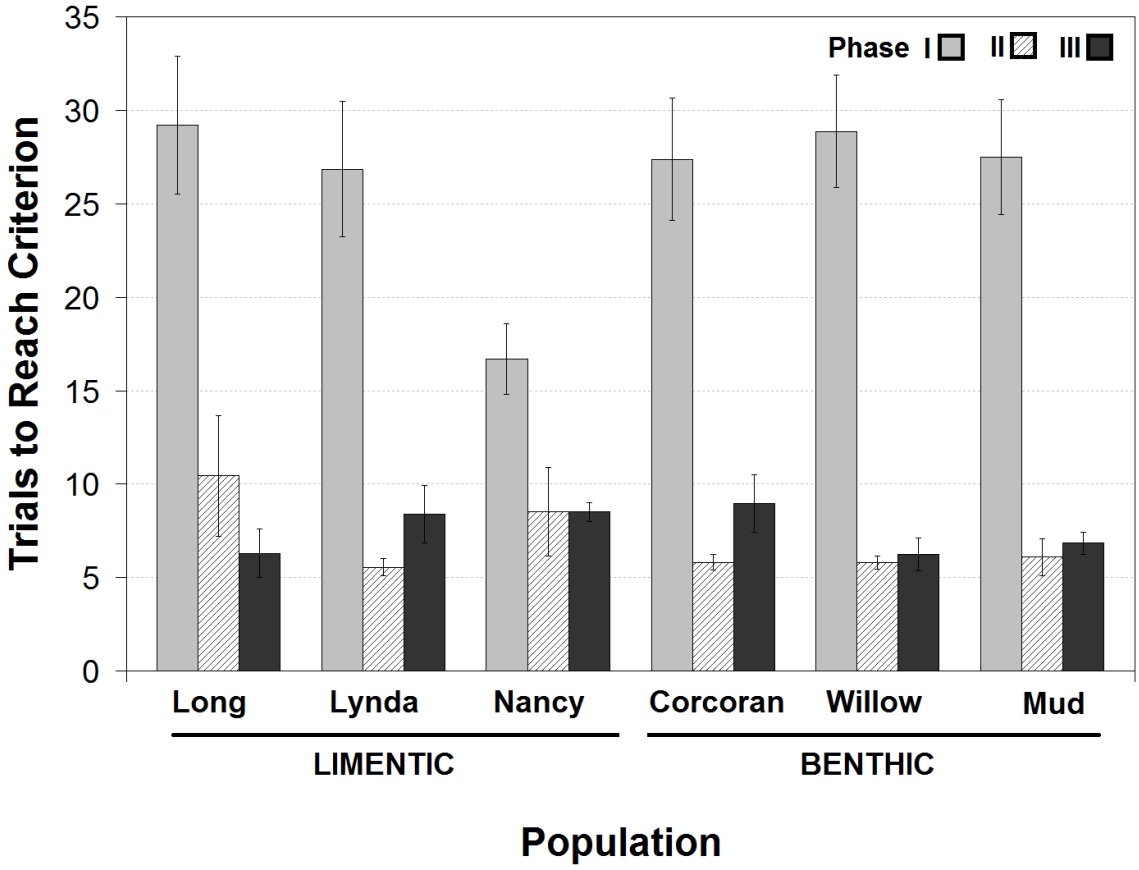
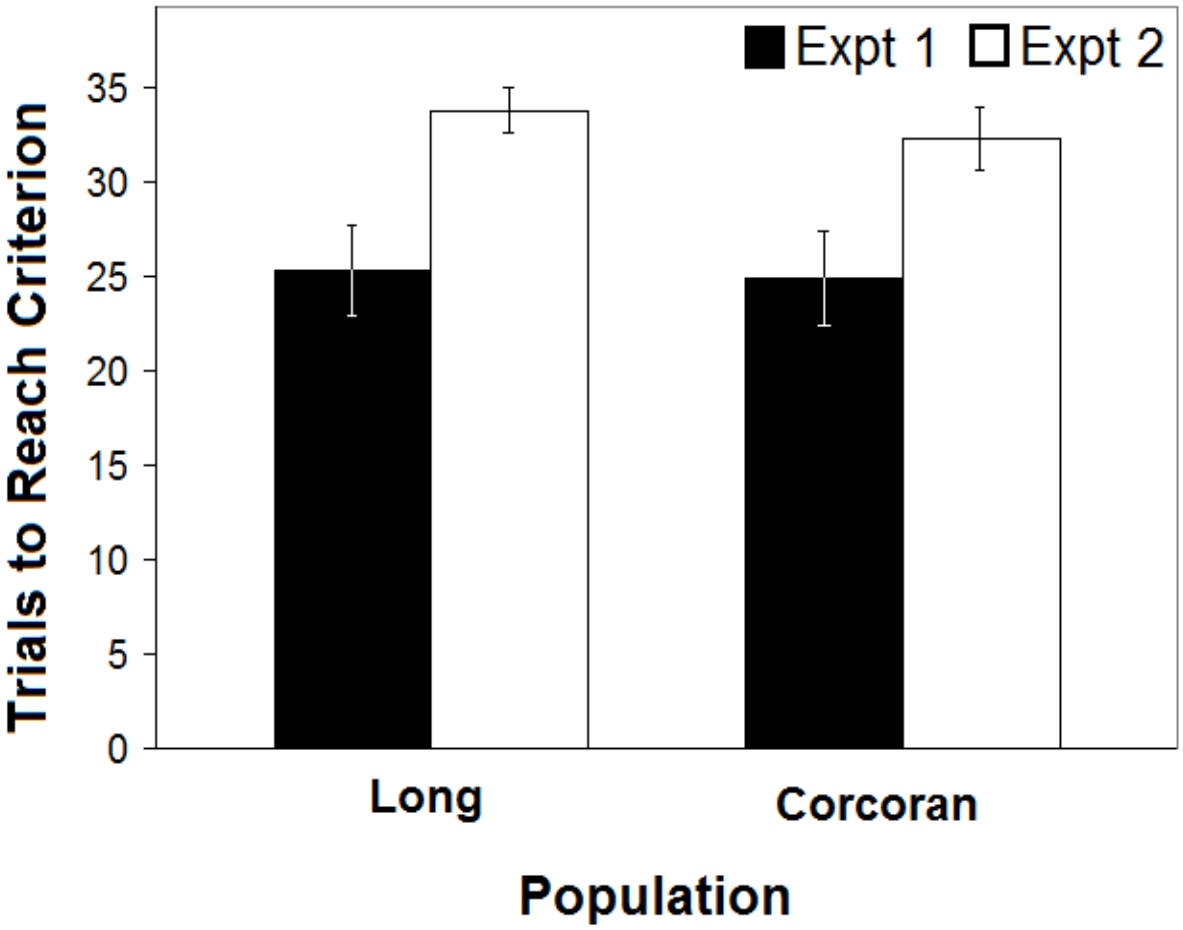


Figure 4. Mean number and standard errors of trials to reach the criterion (Experiment 2). The criterion was nine out of ten trials with correct room choices. Data for Corcoran (benthic) and Long (limnetic) lake samples are displayed. Corresponding values of their counterparts from Experiment 1 are shown for comparison.



Chapter 2

Variation of Spatial Learning of the Threespine Stickleback (*Gasterosteus aculeatus*) in Relation to Inferred Ecology and Experience

ABSTRACT

Spatial learning, the ability to use features in space to navigate within an environment, is important in a variety of animal behaviors. Using a T-maze, the relationship between inferred ecology, ancestry, and experience on spatial learning was investigated in ecologically diverse threespine stickleback fish (*Gasterosteus aculeatus*). In Experiment 1, five benthic (littoral) and five limnetic (open water, planktivore) stickleback populations were compared. Benthics, which feed on the bottom in shallow, structurally complex habitats, exhibit superior spatial learning than limnetics, and these differences cannot be explained by differences in boldness, exploratory behavior, activity level, or other performance variables that are independent of spatial learning. In Experiment 2, a sea-run population (Rabbit Slough) was used to infer the ancestral condition for spatial learning in stickleback. Lab-reared sea-run fish were raised in spatially complex or simple aquaria. While no differences were detected between rearing treatments, a subset of fish from each group solved the maze. Thus, because sea-run fish solved the maze whether reared in simple or complex environments, spatial learning was probably well developed in the sea-run ancestor but the relative contributions of inheritance and experience remain unclear. Therefore, spatial learning has likely been retained in freshwater populations as an adaptation for foraging in shallow, structured microhabitats (e.g., the littoral zone) but reduced in limnetic populations. In Experiment 3, the importance of experience on spatial learning in a freshwater population was

investigated in lab-reared fish from one benthic lake. Lab-reared fish performed poorly compared to their field-caught counterparts, indicating that experience is probably important in development of spatial cognition in individuals from freshwater populations.

INTRODUCTION

Spatial learning is the ability to use visual information concerning features in space to navigate within an environment (Gallisel 1989; Hughes and Blight 1999). In fishes, spatial learning is employed for foraging (Hughes and Blight 1999), predator evasion (Aronson 1951, 1971; Markel 1994; Burns and Rodd 2008), territoriality (Lamanna and Eason 2003), and migration (Fukumori et al. 2010). In mammals and birds, it is associated with food caching (Gaulin and FitzGerald 1986, 1989; Sherry and Duff 1996), maintaining a homerange (see Sherry 1998; Spritzer et al. 2005), and migration (Healy et al. 2005). Although proximate mechanisms underlying spatial learning have been intensely investigated in fishes (Salas et al. 1996a, b; Vargas et al. 2000, 2009; Rodríguez et al. 2002; Odling-Smee and Braithwaite 2003a), its evolution in fishes remains poorly studied. The threespine stickleback fish (*Gasterosteus aculeatus*) species complex has undergone spectacular adaptive radiations since deglaciation of the northern hemisphere, repeatedly producing populations with divergent phenotypic traits. In the current analysis, I show that predictable differences in spatial learning ability are associated with divergence of foraging mode in derived lake populations of this species complex. A separate experiment with laboratory-reared fish from one sea-run (anadromous) population suggests that spatial learning was probably present in the ancestor. Finally, comparison of spatial learning in field-caught and lab-reared specimens from one benthic population indicates that spatial learning is influenced by phenotypic plasticity.

As in birds and mammals (reviewed in Sherry 1998), teleost fishes are able to find a goal using at least three different learning strategies while foraging (López et al. 1999), and individuals can use different learning strategies under different conditions (see Hughes and Blight 1999). Fish can use a single visual landmark as a beacon ("cue learning", *sensu* Salas et al. 1996a; also see Durán et al. 2008) or apply an algorithm, such as learning a sequence of turns (i.e., "turn discrimination," *sensu* López et al. 2000; see also Salas et al. 1996b) to reach a goal. Unlike these strategies, spatial learning employs geometrical features and reciprocal relationships among several nearby (local) and distant (global) visual landmarks to guide navigation (see Gallisel 1989; López et al. 1999; Jacobs 2003). Spatial learning is also associated with the size of the hippocampus of birds and mammals (Healy et al. 2005) and is processed within the dorsolateral region of the telencephalon in fishes (Salas et al. 1996a, b; Vargas et al. 2000, 2009; Rodríguez et al. 2002).

The threespine stickleback (*Gasterosteus aculeatus*) is an excellent model to study the evolution of spatial learning. Its biology and evolution have been studied extensively (Wootton 1976; Bell and Foster 1994; Östlund-Nilsson et al. 2007). It is primitively marine or anadromous but has repeatedly colonized and adapted to diverse freshwater habitats (Bell 1995). Because resident freshwater populations evolved from anadromous stickleback, which appear to be phenotypically conservative (Bell et al. 2009) and geographically homogeneous (Bell and Foster 1994), anadromous populations can be used to infer ancestral spatial learning abilities. In derived lake populations, adaptation to different foraging demands has resulted in predictable ecological and phenotypic divergence among stickleback populations. Benthic (bottom feeding) and limnetic (open-water planktivore) stickleback populations represent extremes along a dietary continuum (Schluter and McPhail 1992). Benthic stickleback prey on invertebrates on highly

structured, shallow lake bottoms, and limnetic populations feed above deep, open waters on plankton. Benthics and limnetics are strongly divergent for foraging behavior and morphology (Schluter and McPhail 1992). Compared to benthics, limnetics have longer, narrower snouts, more dorsal and anal fin rays, more and longer gill rakers (McPhail 1984, 1992,1994; McKinnon and Rundle 2002), more teeth (Caldecutt et al. 2001), greater armor (Vamosi 2002), a more streamlined body (Lavin and McPhail 1985, 1986; Walker 1997; Aguirre 2007) and head (Willacker et al. 2010) form, and when viewed from above, a more triangular dorsolateral region of the telencephalon (Park and Bell 2010). Limnetics can also forage more effectively on plankton than benthics (Bentzen and McPhail 1984) but have shorter memory for handling prey items (Mackney and Hughes 1995). In addition, compared to limnetics, benthics cannibalize young defended by conspecific males (Foster 1994), exhibit less conspicuous courtship behavior (Foster 1994), and show more male-male aggression (Scotti and Foster 2007).

Benthic-limnetic species pairs from lakes in British Columbia may exhibit spatial learning differences (Odling-Smee and Braithwaite, 2003a; Odling-Smee et al. 2008). While sympatric ecotypes did not differ in their propensity to use local visual landmarks in a spatial task, benthics made fewer errors and learned to solve the task sooner than limnetics (Odling-Smee et al. 2008). These results are consistent with the expectation that spatial learning is an adaption for foraging in shallow, structurally complex habitats with numerous landmarks and stable food locations (Odling-Smee et al. 2008). The current study extends these findings by using independently derived allopatric freshwater populations from a geographically distant region - Cook Inlet, Alaska. Although ecological and morphological differences between allopatric benthic and limnetic ecotypes are not as extreme as sympatric benthic-limnetic species pairs (Schluter and McPhail 1992), sympatric benthic-limnetic species pairs occur in only a

handful of lakes (McPhail 1994; Gow et al. 2008) and have not been observed in Alaska (M.A. Bell personal communication). Therefore, ecological differences among derived, allopatric, lake populations are a better representation of the threespine stickleback radiation as a whole.

Allopatric freshwater populations from five benthic and five limnetic populations in Cook Inlet, Alaska were compared to explore the relationship between foraging mode and spatial learning. To increase the chance of detecting a difference in spatial learning, populations with extreme differences were chosen. Populations with the same putative ecotype came from different drainages because similar populations from different drainages are unlikely to share derived characteristics due to common ancestry. While the term "generalists" was used by Park and Bell (2010) to describe a set of Alaskan lake populations with intermediate or extreme planktivore traits, the five populations with the most extreme limnetic traits were selected for this study. Compared to benthics, these populations generally occur in deeper lakes and have shallower body (Aguirre 2007) and skull shapes (Willacker et al. 2010), greater gill raker numbers (see Park and Bell 2010), and more planktivorous dental microwear (Purnell et al. 2006) and foraging behavior (S.A. Foster and J. Baker personal communication) (Table 1). Furthermore, many of the selected planktivore populations have been classified as limnetics by other researchers (see Aguirre 2007; Wund et al. 2008; Willacker et al. 2010). Therefore, the benthic and limnetic populations used in the current study represent opposite poles of a benthic-limnetic continuum for lake populations in Cook Inlet, Alaska.

Fish from anadromous population that breed in Rabbit Slough, Cook Inlet, AK were reared in structurally contrasting environments to infer the ancestral condition for spatial learning. If spatial learning was an ancient trait, it should be present in contemporary sea-run populations. Although the Rabbit Slough population may not represent all sea-run populations,

there is apparently little morphological variation among sea-run threespine stickleback populations worldwide (Bell and Foster, 1994; Walker and Bell, 2000; Colosimo et al. 2005), suggesting limited ecological heterogeneity. The fossil record also indicates that morphological traits of marine threespine stickleback have not changed considerably in the last 13 million years (Bell 1994; Bell et al. 2009).

Experience is expected to be important to spatial learning in threespine stickleback (see Odling-Smee et al. 2008). Learning differences detected using field-caught pond and river stickleback populations (Girvan and Braithwaite 1998, 2000; Odling-Smee and Braithwaite 2003b) disappeared when experiments were repeated with lab-reared fish (Girvan and Braithwaite 2000). Thus, Rabbit Slough fish grown in a structurally complex environment should be better spatial learners than those reared in a structurally simpler environment. The importance of experience on spatial learning in a freshwater population was studied using laboratory-reared fish from Corcoran Lake (benthic). Compared to field-caught fish from this population, lab-reared counterparts were expected to perform poorly.

I used both local and global visual landmarks during spatial learning experiments (see Rodríguez et al. 1994; Salas et al. 1996a, b), but cue learning depends on only local landmarks (see Warburton 1990; Girvan and Braithwaite 1998). Because it can prove difficult to distinguish between spatial or cue learning in experimental subjects if only local landmarks are used, I employed a "spatial constancy" (see Salas et al. 1996a) approach that randomized local landmarks but kept a constant spatial relationship between global cues and the food reward. Although spatial learning has been demonstrated in numerous distantly related fish species (Aronson 1951, 1971; Markel 1994; Salas et al. 1996a, b; Hughes and Blight 1999; de Perera

2004), there has never been an intra-specific study of ecotypic variation in spatial learning that distinguished cue learning and turn discrimination.

MATERIALS AND METHODS

Collection of Field-Caught Fish

Threespine stickleback were collected using 3.18 mm or 6.36 mm mesh, unbaited Gee minnow traps set overnight in heterogeneous microhabitats submerged in less than 1 m depth within 3 m of shore. Ambient water temperature was between 15° and 20° C. Live threespine stickleback were used either for experimental study (Experiment 1) or as parents for crosses to produce laboratory-reared progeny (Experiments 2 and 3). Sampling, husbandry, and treatment of all experimental subjects were approved by the Alaska Department of Fish and Game and by the Institutional Animal Care and Use Committees (IACUC) at the University of Alaska Anchorage and Stony Brook University.

Production of Laboratory-Reared Fish

For Experiments 2 and 3, crosses were performed at the University of Alaska Anchorage. Ten sexually mature fish of each sex from Rabbit Slough (Experiment 2) or Corcoran Lake (Experiment 3) were anesthetized in MS-222 and used in a *mass cross*. Sperm were obtained by slitting the abdomen of ten males and placing the excised testes into a few drops of 3 ppt artificial seawater in a plastic culture dish. The testes of all ten males were minced and mixed with fine forceps to produce a heterogeneous mixture of sperm. Eggs were obtained from ten females by gently squeezing the abdomen to express the eggs into a few drops of 3 ppt artificial seawater in a plastic culture dish. Sperm were pipetted from the minced testis preparation onto

the eggs. A fertilization membrane was observed in the vast majority of eggs within a few minutes, indicating successful fertilization. Eggs were washed repeatedly by pipetting 3 ppt artificial seawater over them and removing the fluid, testis debris, and ovarian mucous. Assuming that the sperm from all testes had equal potency and that embryo mortality was random, random sampling of embryos from a mass cross should include a mixture of individuals with diverse genotypes that are representative of the source population.

The eggs were kept in plastic culture dishes with 3 ppt artificial seawater and transferred to an incubator maintained at 19º C. Within three days after fertilization, they were transferred to 35 or 50 ml vials nearly filled with 3 ppt artificial seawater at 19º C, chilled to 5º C on ice, and shipped in a styrofoam box with freezer packs to Stony Brook University. Approximately 40 random eggs were transferred into each of five plastic culture dishes with 3 ppt seawater and placed into an incubator at 19º C. Water was changed daily. Three to five days after the fry hatched and their yolk sacs were absorbed, they were fed live brine shrimp nauplii daily. Fourteen day-old fry were transferred from culture dishes to specialized spatially complex (SC) or spatially simple (SS), 60 l aquaria for Experiment 2, or to bare 60 l aquaria for Experiment 3 (see below). Once all lab-reared fish reached about1 cm standard length (i.e., distance from tip of snout to end of vertebral column), they were switched to a daily diet of thawed, frozen, adult brine shrimp. Further details of rearing specific to each experiment are explained in their experimental descriptions below.

Maze Apparatus

A four-arm maze similar to that used by Odling-Smee and Braithwaite (2003b) was constructed from 1 cm thick black plexiglass (Figure 1). Each arm was 30 cm long, 10 cm wide,

and 20 cm high. Vertical grooves cut along the height of each arm near their intersection made it possible to close any arm with a 3 mm-thick, black plexiglass wall to produce the T-maze. Two of the three remaining arms located 180° apart in the T-maze had 3 mm-thick black plexiglass walls with 51 x 25 mm cut-out doors in the center. Each wall was located 15 cm away from the end of the arm, creating a room in which food could be placed (see Figure 2). A sliding trap wall located 15 cm away from the end of third arm in the T created the starting area. This trap wall could be lifted by the observer by pulling on a clear, monofilament thread. The maze was submerged in a 100 cm diameter x 30 cm-high circular pool filled with 3 ppt artificial seawater to a depth of 19 cm.

Experimental Enclosure (Cage)

A 92 cm long, 78 cm wide, and 131 cm high steel framed cage lined with opaque white shower curtains was constructed to minimize disturbances occurring outside the maze (Figure 1). A fluorescent lighting fixture (Lights of America, Model No. 8045: 118V 60Hz 70W 8A, Walnut, California) with one bulb (Sylvania Gro-Lux, 40W, Danvers, Massachusetts) was attached along each length of the cage to the top frames. A 51 cm tall video camera stand that rested atop the cage was constructed from PVC, plastic, and wood. The distance from the camera lens to the top of the maze was 119 cm. Entry into the cage was through a slit between overlapping shower curtains along one side of the cage.

Pre-training and Housing of Experimental Subjects

Pre-training trials were administered to familiarize all fish with the experimental apparatus. During pre-training trials, all four arms were accessible and no doors were used.

Thawed frozen adult brine shrimp were placed on a plastic culture dish at the end of all four arms. Fish were motivated by food restriction for 24-36 hrs prior to pre-training and fed only in the maze. Each population was divided into two sets of similar sample size. Each set of fish was allowed to swim together freely for one hour during a pre-training trial. One pre-training trial was administered every other day for up to three days.

After the final pre-training trial, each fish was placed individually in separate 13 cm x 8 cm x 26 cm (0.5 cm thick) plexi-glass holding compartment to keep track of it during the experiment. Twelve of these compartments were suspended side-by-side in each of up to eight sixty-liter aquaria with 3 ppt artificial saltwater filtered continuously with a sponge filter (Hydro-Sponge II, Aquarium Technology, Inc., Decatur, Georgia) and hanging power filter (Aquaclear 30 power filter, Rolf C Hagen Corp., Mansfield, Massachusetts). A circular hole (radius = 37 cm) covered with 1 mm-mesh located on either 13 cm x 8 cm side of a compartment allowed the filtered water to pass through all the compartments in an aquarium constantly. Each fish was housed in the same compartment through the experiment. The compartments were randomly rearranged among the aquaria on a daily basis to eliminate potential confounding effects of water quality differences across aquaria. All aquaria were maintained on a 12 hour light: 12 hour dark photoperiod at 18°C and 3 ppt artificial seawater.

Experimental Trials

The T-maze was constructed by permanently closing off one arm, creating two rooms that were 180° apart, and creating the starting area in the remaining arm. During all experimental trials, three conspicuous global (distant) visual extra-maze cues were located outside of the maze but within the experimental enclosure to facilitate spatial learning ability (Figure 1). A 71.5 cm x

19.5 cm piece of blue poster board was located on the shower curtain opposite the enclosure entrance, a 27 cm x 18.5 cm black and white-banded board was located to the right, and a 27 cm x 18.5 cm solid black board was to the left of the entrance. Blue and black colors were used because threespine stickleback can distinguish between them (Rowe et al. 2004). The video camera was connected to a TV monitor and a VCR that was used to record all trials.

Before each trial, four thawed, frozen, adult brine shrimp were placed into the food reward room. Every experimental series was subdivided into two sets of fish to test for side bias in the maze. The fish in one set were given their food reward in the room to the left of the starting area during the experiment (i.e., left-assigned fish), while fish from the other set were fed to the right (i.e., right-assigned fish). Subjects were motivated by food restriction for 24-36 hrs between trials. The order of subjects run in the maze was randomized among trials using a random number generator.

Individual fish were expected to adopt spatial learning to the exclusion of cue learning or turn discrimination by randomizing the location of the starting area by 180° between trials (Figure 2). A random number generator was used to select the order in which each of the two orientations was used, except that no more than three consecutive trials with the same orientation were allowed. When the starting area was rotated 180°, the pool and maze within it were rotated together to ensure that no local (nearby) visual intra-maze cues (e.g., scratches on the maze or pool, conspicuous gravel) contributed to learning the food reward location using cue learning. However, because the spatial relationship of the global (distant) visual extra-maze cues and the food reward never changed during the entire duration of an experiment, subjects had to use spatial learning to solve the maze. To eliminate olfactory cues, half of the water in the maze was replaced with water from the pool after every subject was run, and a complete water change for

the pool and maze was done after each trial. Although non-visual information could still have been used, olfactory (Girvan and Braithwaite 1998; also see Odling-Smee and Braithwaite 2003b; Park in preparation) and auditory cues (Park in preparation) appear to be unimportant to stickleback to navigate in the T-maze.

At the start of every experimental trial, a single fish was placed in the starting area for 60 s, after which the trap wall was raised. The time that each fish first left the starting area was recorded (referred to hereafter as "starting time", except for trial 1, see below). The fish was allowed to swim freely through the maze. As an indicator of "activity level" (except for trial 1, see below), total movement (also called ambulation) in the maze was recorded as the sum of entries a fish made into either arm or back into the starting area. A trial was completed when the fish entered the food reward room and fed, after which the fish was left for an additional 5 minutes before being gently netted and returned to its individual holding compartment. The time interval between first leaving the starting area and entering the food reward room (hereafter referred to as "food time") was recorded. All performance measures were based on the passage of the caudal peduncle (see Hughes and Blight 1999) through the relevant plane for the starting area, base of either T-maze arm, or door of a room.

Six out of seven trials in which the subject found the food reward room without entering the unrewarded room was the criterion for spatial learning. This criterion occurs within a range of successful criteria used by Odling-Smee and Braithwaite (2003b) and Odling-Smee et al. (2008) to investigate spatial learning in threespine stickleback. Any correct choices made during the first two trials were excluded from the criterion because these could have occurred by chance. A maximum of 50 trials per fish was used. Comparable studies with stickleback have used 45 trials as a maximum limit (see Odling-Smee and Braithwaite 2003; Brydges et al. 2008).

A trial ended if a fish did not leave the starting area within 10 min or did not find the food reward within 10 min after initially leaving the starting area. In either case, the fish was "encouraged" by gently prodding it with a net to the food reward. Encouragement using this approach is a standard procedure employed in behavioral studies with fishes (see Salas et al. 1996a), and all fish that had been encouraged were observed feeding in the food reward room before being transferred to their holding compartment. Whether they were encouraged or found the food reward room themselves, all fish fed only in the maze to maintain their motivation to reach the criterion. A limit of 10 minutes per trial to solve the maze was used in a similar study using fishes (Brown and Braithwaite 2005), and it did not bias results. All subjects completed the first two trials without necessitating encouragement, and so, it is unlikely that encouragement biased some fish not to solve the maze at the start of an experiment.

Preliminary studies indicated that fish reaching ten encouraged trials were unable to solve the maze, and for logistical reasons, further experimentation was terminated for fish that were encouraged ten times. Observers were not given any information about the ecotype or source population while behaviors were recorded.

Trial 1: Boldness and Exploratory Behavior

The performance of each fish during its first trial in the T-maze was used to estimate differences in boldness and exploratory behavior between ecotypes (Experiment 1), rearing treatment (Experiment 2), or field-caught and lab-reared samples (Experiment 3). During this trial, an individual fish was exposed for the first time to a starting area, moving trap wall, and a room with a door at either end of the arms that were 180° apart. Subjects were acclimated for 60 s before the trap wall was opened.

Boldness is "the willingness to accept risk in return for potentially higher foraging or reproductive gain" (Ward et al. 2004). Bolder individuals are more likely to explore novel surroundings (see Budaev 1997), potentially biasing them to learn about their surroundings sooner than shy (i.e., low boldness) individuals (see Burns and Rodd 2008). Freezing is a behavior used to determine tendency for boldness in fishes (Walsh and Cummins 1976; Budaev 1997; Burns 2008). It is a reliable indicator of boldness in stickleback because it is a strategy used to evade detection by predators (Wootton 1984; Huntingford and Coyle 2007). Boldness was measured as the residual of the total number of freezes regressed on the log of the time interval between when a fish's caudal peduncle left the starting area and first entered a door into a food reward room.

Ambulation in a novel environment is a reliable indicator of exploratory behavior (Walsh and Cummins 1976; Gervai and Csányi 1985; Budeav 1997; Burns 2008). Subjects that explore by re-visiting areas may generate a better internal representation of their new environment than those that do not (Capaldi et al. 2000; Eilam et al. 2003). Ambulation is typically measured as the time spent in motion (Budeav 1997; Burns 2008) or the number of times a subject crosses regular spatial intervals (Gervai and Csányi 1985). In the current study, ambulation was recorded as the number of times the subject entered either arm or returned to the starting area during trial 1. Because many aspects of the maze (e.g., trap wall, rooms with doors, extra-maze cues) were novel to subjects during this trial, ambulation was a better proxy for exploratory behavior and not for activity level. Activity level is typically measured under conditions in which subjects have had considerable experience or are under low stress (Burns and Rodd 2008; also see below).

Emergence tests measure how willing the subject is to leave a starting area (Gervai and Csányi 1985; Burns 2008). While latency to emerge has been considered a measure of boldness

(Brown and Braithwaite 2004; Brown et al. 2005), it may more accurately reflect a conflict between shyness and willingness to explore (Burns 2008). Therefore, in the current study, “latency to emerge" was used as a composite measure of shyness and exploratory behavior. It was recorded as the time that a fish's caudal peduncle left the starting area and entered the maze for the first time (see Odling-Smee et al. 2008). During trial 1, the time that a fish left the starting area after the trap wall was raised could be treated as an emergence test because all fish emerged slowly out of the starting area as if they were inspecting their surroundings, and a majority of them returned to the starting area before entering a room (Expt 1, 84%, n=135; Expt 2, 78%, n=18; Expt 3, 88%, n=26). Therefore, it is reasonable to assume that subjects treated the starting area as an artificial refuge and the newly exposed area of the maze as a novel environment.

Criterion Trials: Starting Time, Food Time, and Activity Level

Performance differences among or within samples of successful learners may provide insight into behaviors that contribute to spatial learning differences. Fish that solved the maze were analyzed further to determine whether performance differences were present between ecotypes (Experiment 1), rearing treatment (Experiment 2), or field-caught and lab-reared samples (Experiment 3). The six successful (out of seven) criterion trials for starting time, activity level, and food time were analyzed (Table 2). This dataset included more-or-less similarly experienced individuals that did not make wrong door errors. The starting time was the time that each fish initially left the starting area. Activity level was recorded as the sum of entries a fish made into either arm or back into the starting area. Fish that reached the criterion are experienced and presumably under less stress in the T-maze than during trial 1. Therefore, total movement during criterion trials should be more indicative of activity level and not of

exploratory behavior. Finally, food time was recorded as the time interval between first leaving the starting area and entering the food reward room. All measurements were based on passage of the caudal peduncle.

Experiment 1: Ecotypic Comparisons of Field-Caught Samples

Field-caught samples from five benthic and five limnetic populations were used to test for ecotypic differences in spatial learning. Due to high mortality of senescing field-caught adults in captivity, pre-reproductive one-year old threespine stickleback were collected, employing methods described above. In June 2006, I sampled threespine stickleback from Corcoran, Walby, and Willow lakes, which contain benthic stickleback, and from Long, Lynne, and Matanuska lakes, which contain limnetic stickleback. In June 2008, I sampled Mud and Tern lakes, which contain benthic populations, and South Rolly and Stormy lakes, which are inhabited by limnetics. Table 1 and Park and Bell (2010) present dietary, phenotypic, geographic, and ecological details for these populations and lakes. Stickleback from ecotypically similar lakes came from lakes that drain independently to Cook Inlet to maximize the likelihood that they were derived independently from an anadromous ancestor (Table 3; Figure 2).

Live pre-reproductive fish were transported to the University of Alaska Anchorage and kept in aged tap water in outdoor pools for 24-48 hours and prepared for shipping. They were placed into shipping bottles that were aerated with an air stone and cooled down to 5-8° C. The bottles were sealed and placed into an ice chest with freezer packs and shipped overnight to Stony Brook University. Upon arrival, the fish were acclimated for water temperature over several hours and then transferred to 60 l aquaria. The fish were fed once per day with thawed frozen adult brine shrimp. As a standard procedure, after fourteen days of captivity in the lab, all

fish were treated for potential pathogens that typically compromise the welfare of field-caught stickleback held in captive conditions for several months. No mortality was observed during medical treatment. Experiments with these fish commenced three weeks after their arrival to Stony Brook University. Attrition did not occur during pre-training but two fish from Stormy Lake (limnetic) and three fish from Tern Lake (benthic) did not survive before completing the experiment (Table 3). These data were not omitted because statistical methods that can account for data lost due to mortality before conclusion of an experiment (i.e., progressively censored data) were used to compare spatial learning differences (see Statistical Analyses). Final sample sizes used in experimental trials are listed in Table 3. It is very unlikely that welfare differences among samples were present during trials because all subjects were housed under identical conditions and shared the same 3 ppt artificial saltwater (see Pre-training and Housing of Experimental Subjects). Fish that survived were considered healthy by observing their overall alertness, presence of erect complete fins, and active feeding in the maze. Therefore, the loss of five fish in Experiment 1 was probably due to random factors beyond the control of investigators.

Experiment 2: Plasticity in a Sea-Run Sample

Anadromous threespine stickleback from Rabbit Slough (see Aguirre et al. 2008; Park and Bell 2010 for geographic and ecological details for this site) were used to infer the ancestral state of spatial learning. Only laboratory-reared fish could be used because of high mortality of senescing field-caught adults in captivity and the lack of known sites from which to collect a large sample of wild sea-run pre-reproductives. In June 2006, ten sexually mature sea-run threespine stickleback were collected from Rabbit Slough and used in a mass cross to generate

lab-reared fish, employing methods described above (see Production of Laboratory-Reared Fish). Because learning strategies in stickleback depend greatly on experience (Girvan and Braithwaite 2000), 14 day-old fry were transferred from culture dishes to either spatially complex (SC) or spatially simple (SS) aquaria (Table 3; Figure 2). Both types of aquaria were constructed by fastening a 29 cm x 29 cm black plexiglass platform, dividing it into upper and lower halves along one half of its length; the opposite half of the aquarium remained open. In the SC aquarium, the plexiglass platform had 15 cm long frayed white polypropylene rope protruding above and below the platform, creating four equal-size quadrants. In the SS aquarium, no rope was used. A bare platform was placed in the SS aquarium in case there were any unanticipated effects of having the platform during rearing. Each aquarium was filtered using a sponge filter (Hydro-Sponge II, Aquarium Technology, Inc., Decatur, Georgia) and maintained on a 12 hour light: 12 hour dark photoperiod at 19º C with 3 ppt saltwater.

Both types of aquaria were isolated by a white shower curtain to eliminate external visual cues during rearing. A specialized feeding apparatus was constructed for both rearing treatments using white polyvinyl chloride pipes so that all fish fed off of the horizontal surface of the bottom or the platform. One 2.2 cm diameter, 28 cm long pipe was attached to a 2.2 cm diameter, 12 cm long pipe so that their tops were adjacent but their bottoms were 16 cm apart. The top of the apparatus was held together with a 6.6 cm diameter, 2.2 cm long pipe, and silicone glue. The apparatus allowed food to be inserted into either of the smaller pipes to deposit the food onto the platform or aquarium bottom without the fish knowing in advance where it would be delivered. All fish were forced to forage off the bottom to eliminate any confounding effects of variation in food location in the water column. In the SS aquarium, food was deposited simultaneously on both the platform and aquarium bottom. In contrast, in the SC aquarium, all

food was deposited only at one randomly determined level, and brine shrimp liquid with no food was deposited in the remaining tube. Therefore, fish in the SC aquarium had to negotiate the frayed white polypropylene rope above and below the platform to find their food on a daily basis. Experimentation with these fish commenced at one year of age.

Experiment 3: Field-Caught and Lab-Reared Corcoran Lake Fish

The contribution of experience in the wild to spatial learning was tested using laboratory-reared fish from Corcoran Lake (benthic) because this population had a large number of successful spatial learners in Experiment 1 (Table 3; Figures 2 and 3). Lab-reared fish were raised in a bare aquarium, and their performance in the T-maze was compared to that of their field-caught counterparts from Experiment 1. While this design may be inferior to comparing lab-reared fish raised in spatially contrasting aquaria, findings from Experiment 2 (See Results and Discussion) and Girvan and Braithwaite (2000) indicate that artificial complexity in an aquarium is probably not effective in generating spatial learning abilities.

In June 2007, ten sexually mature threespine stickleback of each sex were collected from Corcoran Lake and used in a mass cross (see Production of Laboratory-Reared Fish) to produce the lab-reared fish. Fourteen day-old fry were transferred from culture dishes to bare 60 l aquaria on a 12 hour light: 12 hour dark photoperiod at 19º C and 3 ppt salinity. Experiments with these fish commenced after they reached one year of age.

Statistical Analyses

Each experiment was analyzed separately. Statistica version 9.1 or Biomstat version 3.30q were used to test for differences in spatial learning and several other measures of

performance between ecotypes (Experiment 1), rearing treatment (Experiment 2), or samples (Experiment 3). ANOVA tests were used if data could be transformed such that sample residuals conformed to a normal distribution (see Sokal and Rohlf 1995; Table 2). Fish assigned food in the room to the left or to the right of the enclosure entrance were tested for side bias whenever it was appropriate.

For each experiment, subjects could be placed into one of three categories (Table 3): fish that solved the maze (S fish), did not solve the maze within 50 trials (NS fish), or were encouraged ten times before reaching 50 trials (E fish). Spatial learning was inferred using two analyses. Trials to reach the criterion is time-to-event (or waiting time) data which are not expected to be normally distributed, and thus, tests that compare waiting time data were used to analyze samples for this variable (see Lee and Wang 2003). These tests do not make any assumptions about the waiting time distribution for a sample. In the second analysis, a G-test of independence tested whether the proportion of fish that solved the maze statistically differed from the number of those that did not (i.e., NS and E fish).

All subjects were used for tests of boldness, exploratory behavior, or latency to emergence (i.e., boldness-exploratory behavior conflict) (Table 2). To measure boldness, ANCOVA was used to compare relative number of freezes with log of the time until first entry into a door as the covariate. Nested ANOVA was used to test exploratory behavior and emergence times.

Fish that solved the maze were investigated further for ecotypic differences in other maze performance measures (Table 2). Results for their six successful (out of seven) criterion trials for starting time, food time, and activity level were analyzed using repeated-measures ANOVA to

compare ecotypes (Experiment 1), rearing treatments (Experiment 2), or field-caught and lab-reared samples (Experiment 3).

RESULTS

Validation of Spatial Learning Criterion

The 2006 dataset of Experiment 1 provided the largest and most complete dataset and was employed to validate use of the criterion (i.e., correctly choosing the food room in six out of seven trials) for learning. Data for the first 20 trials was collected for all subjects because this number of trials is sufficient to detect learning improvement in a spatial task (see Salas et al. 1996b). Fish that eventually solved the maze within 50 trials were analyzed separately from those that did not solve it (i.e., E and NS fish). The relative proportion of correct choices made by fish that achieved this criterion was plotted against trial number for the first 20 trials (Figure 4). If subjects were learning to avoid the unrewarded room in later trials, the proportion of correct choices made by subjects was expected to increase with trial number. Angular transformation (i.e., the inverse sine of the square-root of the proportion) was used for proportional data (Sokal and Rohlf 1995). The slope of the regression of transformed proportions on trial number was greater than zero for fish that achieved the criterion (N=56, slope$\pm$SE=0.396$\pm$0.165, $F_{1,18}$=5.773, $p<0.05$) but not for fish unable to solve the maze (N=40, slope$\pm$SE=-0.062$\pm$0.243, $F_{1,18}$=0.066, p=0.800). A greater proportion of subjects that achieved criterion avoided the unrewarded room during the first 20 trials. Thus, using six out of seven trials without a wrong-door error is an effective criterion for learning to find food in the maze.

Experiment 1

This experiment was used to test for an association between spatial learning and ecotype. Transformations failed to normalize the sample residuals for trials to reach the criterion and tests that compare waiting time (or time-to-event) data were used. Fish that did not reach the criterion (i.e., NS and E fish) were designated the maximal 50 trials but treated as singly censored data in the analysis. For individuals that did not survive before reaching criterion (i.e., A fish), which only occurred in Experiment 1 (see Table 3), the trial number reached just prior to attrition was incorporated into the analysis as progressively censored data (see Table 3 for sample sizes). A Gehan's generalized multi-sample Wilcoxon test failed to detect differences for waiting time distributions of trials to reach the criterion across benthic (χ_4^2= 2.595, p=0.628, Figure 3) or limnetic (χ_4^2= 2.326, p=0.676, Figure 3) samples. Therefore, results from separate populations were pooled within ecotype. Benthics (N=70) reached the criterion in fewer trials than limnetics (N=70) (Peto and Peto Wilcoxon Test: W=-1.978, p<0.05). More benthic fish (N=38 of 67) reached criterion than limnetics (N=30 of 68), but a G-test of independence revealed that this difference was not significant (benthics, N=67; limnetics, N=68; G_{adj}=2.125, p>0.05). However, the central question of this research can still be answered affirmatively. Although the relative frequency of benthics and limnetics that met the criterion was indistinguishable, the benthics reached criterion sooner. Therefore, benthic foraging fish are superior at spatial learning to locate food than limnetics.

Boldness, exploratory behavior, and latency to emerge could have influenced these results. Unlike trials to reach criterion, these variables did not include any censored data because all fish participated in the first trial. To compare boldness, ANCOVA was used on square-root transformed total number of freezes with the log of time spent between first leaving the starting

area and entering a room as the covariate. No differences were detected among benthic ($F_{4,64}$=1.795, p=0.141) or limnetic ($F_{4,64}$=0.528, p=0.715) samples, and therefore, populations within ecotype were pooled. ANCOVA failed to detect differences between ecotypes ($F_{1,137}$=0.144, p=0.705). To compare exploratory behavior, a three-level nested ANOVA with side⊂population⊂ecotype (i.e., "within" noted hereafter as ⊂ for nested ANOVA) as groups and square-root transformed total entries into maze arms and starting area as the dependent variable was performed. Nested ANOVA failed to detect an effect of ecotype ($F_{1,8}$=0.0014, p=0.971), population ($F_{8,10}$=1.442, p=0.288), or side ($F_{10,120}$=0.386, p=0.951). A three-level nested ANOVA with side⊂population⊂ecotype as groups and latency to emerge (i.e., log of time initially leaving starting area) as the dependent variable also failed to detect an effect of ecotype ($F_{1,8}$=1.835, p=0.213), population ($F_{8,10}$=2.700, p=0.072), or side ($F_{10,120}$=0.985, p=0.460). Thus, generally, boldness, exploratory behavior, or latency to emerge did not differ between populations, the side on which the food was placed, or ecotypes.

To compare performance during criterion trials among successful learners, ecotypic differences for other behavioral measures were tested in fish that achieved the criterion of six correct choices in seven tries. Lake populations were pooled within each ecotype because limited sample sizes did not allow an analysis among populations. A repeated-measures ANOVA with ecotype as the factor and activity level during the six successful criterion trials as the dependent variable was not statistically significant ($F_{1,66}$=1.12, p=0.295). Similarly, a repeated-measures ANOVA did not detect an effect of ecotype for starting time ($F_{1,66}$=1.11, p=0.296) or food time ($F_{1,66}$=0.006, p=0.939). Thus, starting time, food time, or activity level did not differ between ecotypes.

Overall, benthics outperformed limnetics in trials to reach criterion, and boldness, exploratory behavior, latency to emerge, activity level, or other performance measures that are not directly related to spatial learning but might influence its formation cannot account for the differences in maze performance.

Experiment 2

This experiment was designed to infer the role of experience on spatial learning in an ancestral, anadromous population and employed laboratory-reared fish from Rabbit Slough. Again, data transformations did not normalize sample residuals for trials to reach the criterion. A Peto and Peto Wilcoxon test, with results for NS and E fish treated as singly censored data, did not detect a difference between rearing treatments for trials to reach criterion ($W=1.014$, $p=0.311$). A G-test of independence ($G_{adj}=1.321$, $p>0.05$) or multinomial exact test (SC, $N_{Solved}=8$, $N_{Not\ Solved}=5$, $p=0.157$; SS, $N_{Solved}=5$, $N_{Not\ Solved}=8$, $p=0.157$) failed to detect a difference between rearing treatments in the number of fish that solved the maze. A multinomial exact test was used here because it is statistically more powerful than the G-test when sample sizes are small (Conahan 1970).

To compare boldness, I used ANCOVA of the square-root transformed total number of freezes with log of time between first leaving the starting area and entering a room as the covariate. ANCOVA failed to detect a difference between treatments ($F_{1,23}=0.146$, $p=0.705$). A two-level nested ANOVA with side$\subset$treatment as groups was used to compare exploratory behavior and latency to emerge. Using exploratory behavior as the dependent variable failed to detect an effect of treatment ($F_{1,2}=2.359$, $p=0.264$) or side ($F_{2,23}=0.590$, $p=0.562$). Similarly, using latency to emerge as the dependent variable did not detect an effect of treatment

($F_{1,2}$=4.213, p=0.177) or side ($F_{2,22}$=0.550, p=0.584). Thus, boldness, exploratory behavior, or latency to emerge did not differ between rearing treatments.

Differences between rearing treatments for other maze performance measures were tested in fish that achieved the criterion of six correct out of seven consecutive trials to compare performance among successful learners. Repeated-measures ANOVA with activity level as the dependent variable did not detect an effect of treatment ($F_{1,11}$=0.395, p=0.543). Repeated-measures ANOVA also failed to detect an effect of treatment for starting time ($F_{1,11}$=0.440, p=0.519) or food time ($F_{1,11}$=0.415, p=0.533). Thus, starting time, food time, or activity level also did not differ between rearing treatments.

Overall, no differences for spatial learning were detected between rearing treatments. Boldness, exploratory behavior, latency to emerge, activity level, or other performance measures also did not differ between treatments.

Experiment 3

This experiment was performed to investigate the influence of experience on spatial learning in a lake population with a substantial number of proficient spatial learners from Experiment 1. As in previous experiments, transformations did not normalize the sample residuals for trial to reach criterion. Thus, I used a Peto and Peto Wilcoxon test with results for NS and E fish treated as singly censored data. The waiting time distribution of trials to reach criterion for field-caught fish was lower than that of lab-reared fish (W=-2.782, $p<0.05$). A G-test of independence revealed that more field-caught fish (N=10) than lab-reared fish (N=3) solved the maze (field, N=16; lab, N=18; G_{adj}=7.486, $p<0.05$; Figure 5). Thus, using two

independent tests, field-caught Corcoran Lake benthic fish were superior spatial learners than their lab-reared counterparts.

Again, boldness, exploratory behavior, and latency to emerge are potential confounding variables that could have influenced these results. To determine whether boldness could have influenced measures of spatial learning, ANCOVA was used on square-root transformed total number of freezes with log of time between first leaving the starting area and entering a room as the covariate. Lab-reared fish froze fewer times than their field-caught counterparts ($F_{1,31}$=12.617, p<0.01; Figure 6). A two-level nested ANOVA with side⊂sample as groups was used to compare exploratory behavior and latency to emerge. Using exploratory behavior as the dependent variable did not detect an effect of sample ($F_{1,2}$=6.132, p=0.132) or side ($F_{2,30}$=0.360, p=0.701). There was also no significant effect of sample ($F_{1,2}$=3.042, p=0.223) or side ($F_{2,30}$=0.713, p=0.498) on latency to emerge. Thus, generally, boldness, exploratory behavior, or latency to emerge did not differ between field-caught and lab-reared samples.

To compare successful learners, differences between field-caught and lab-reared samples for other maze performance measures were tested in fish that achieved the criterion. A repeated-measures ANOVA with activity level as the dependent variable did not detect an effect of sample ($F_{1,11}$=0.451, p=0.516). Repeated-measures ANOVA also did not detect an effect of sample for starting time ($F_{1,11}$=0.600, p=0.456) or food time ($F_{1,11}$=0.058, p=0.814). Thus, starting time, food time, or activity level did not differ between field-caught and lab-reared samples.

Overall, field-caught fish outperformed lab-reared fish for trials to reach the criterion, and more of them successfully solved the maze. Field-caught fish were also not as bold as their lab-reared counterparts, but these differences in boldness are a conservative departure from equal

boldness in the samples. None of the remaining potential confounding factors, exploratory behavior, latency to emerge, activity level, or other performance measures influenced these results.

DISCUSSION

Spatial learning creates the ability to generate a cognitive map of the environment, which can allow a navigator to create novel routes to a goal (Tolman 1948; reviewed in Jacobs 2003). O'Keefe and Nadel (1978) identified several operational criteria for spatial learning that have been confirmed in fishes (López et al. 1999). The evolution of spatial learning was explored in the current study using populations of threespine stickleback (*Gasterosteus aculeatus*) in an endemic Alaskan adaptive radiation (Bell et al. 1993). Unlike spatial learning, cue learning and turn discrimination are probably ubiquitous among fish species (see Warburton 1990, 2003; López et al. 1999). While others have explored ecological variation of spatial learning in threespine stickleback (see Girvan and Braithwaite 1998, 2000; Odling-Smee and Braithwaite 2003b; Brydges et al. 2008; Odling-Smee et al. 2008), this study is the first to do so while controlling for cue learning and turn discrimination. The present research shows that foraging ecotypes from allopatric freshwater stickleback populations differ in spatial learning performance, that this trait was probably present in the anadromous ancestor, and that it depends on experience.

Independent derivation and phenotypic divergence of numerous freshwater threespine stickleback populations from a common anadromous ancestor allows different lake populations to be used as phylogenetically and statistically independent “evolutionary experiments” (Bell and Foster 1994; Bell 1995), and the ancestral condition for any trait can probably be observed in

anadromous populations from which they evolved. Derived threespine stickleback lake populations sampled for Experiment 1 occur in a recently deglaciated region of southcentral Alaska (Reger and Penny 1996) that was colonized by anadromous ancestors within the last 22,000 years (Bell et al. 1993), and the Rabbit Slough population used in Experiment 2 is a living representative of the anadromus ancestor. Freshwater populations in different river systems are more closely related to adjacent sea-run populations than to freshwater populations in other drainages (Taylor and McPhail 1999, 2000; Aguirre 2007), and therefore, freshwater populations from different drainages are free to evolve independently. Thus, samples were chosen such that ecologically similar populations came for different drainages and must have evolved their similarities, if any, independently from anadromous ancestors.

The T-maze protocol encouraged spatial learning based on global (distant) extra-maze visual cues without employing cue learning or turn discrimination learning strategies. Randomization of the starting area made turn discrimination unreliable. Rotation of the maze apparatus and pool during these trials eliminated the importance of local (nearby) visual intra-maze cues (e.g., scratches on walls, conspicuous gravel), which are preferentially used for cue learning (see Rodríguez et al. 1994; Salas et al. 1996a; Warburton 1990). Sensory modalities other than vision may be used to learn spatially (see Jacobs and Schenk 2003). However, threespine stickleback rely more heavily on visual information than olfactory (Girvan and Braithwaite 1998; also see Odling-Smee and Braithwaite 2003; Park in preparation) and auditory cues (Park in preparation) during experiments using a T-maze. Another potential confounding variable is that the T-maze could have favored benthics over limnetics because subjects were largely confined to a small space and had to make abrupt turns to solve the maze. While we are not the first to use a T-maze to investigate learning differences between benthics and limnetics, it

would be interesting to see if these ecotypes behave differently in a maze that is more open such as a "hole-board" task (c.f. Durán et al. 2008).

Experiment 1 shows that compared to limnetics, benthics require fewer trials to solve the maze, and this difference cannot be explained by boldness, exploratory behavior, and activity level. More benthics than limnetics solved the maze, which is consistent with the need for fewer trials in benthics, but this difference was not statistically significant and may be unreliable. Spatial learning in fishes is used during foraging (Hughes and Blight 1999), predator evasion (Aronson 1951, 1971; Markel 1994; Burns and Rodd 2008), maintenance of territories (Lamanna and Eason 2003), and migration (Fukumori et al. 2010). Odling-Smee et al. (2008) discovered that benthic threespine stickelback were more efficient learners than sympatric limnetics even though the ecotypes did not differ in their propensity to use spatial cues over turn discrimination. They proposed that the greater efficiency of benthics compared to limnetics was due to superior spatial learning, and the current study confirms this contention. Compared to benthics, which live in shallow lakes dominated by littoral habitat, limnetics may still use spatial learning to forage in the relatively limited littoral areas surrounding the deep, open areas of their lakes (see Schluter and McPhail 1992; Odling-Smee et al. 2008; Park and Bell 2010). Interestingly, a few fish in every sample were incapable of spatial learning, suggesting that considerable variation for it persists among stickleback populations. Although this study is the first to identify spatial learning to the exclusion of cue learning and turn discrimination strategies in threespine stickleback, further study is needed to understand individual variation within populations and its causes in natural populations.

To test the possibility that there are performance differences between successful learners in the benthic and limnetic populations, the dataset from only fish that achieved the criterion was

used to compare general activity level as well as the time it took for subjects to leave the starting area and complete the trial. This dataset included fish with comparable experience, and it did not take wrong-door errors into account. There were no differences for any of these performance variables between benthic and limnetic ecotypes. Therefore, while benthics are superior spatial learners, benthic and limnetic spatial learners do not appear to differ in how quickly they left the starting area, found the food reward room, or moved around the maze during a comparable set of trials.

Spatial learning can be distinguished experimentally from cue learning and turn discrimination, and it also differs from them by having a size-dependent neuroanatomical correlate. In birds and mammals, the size of the hippocampus is positively associated with the extent of spatial learning. Birds that cache seeds and migrate use spatial learning and have a larger hippocampus than closely related species that do not (Clayton and Krebs 1994; Healy 1996; Krebs and Davies 1997; Healy et al. 2005). In kangaroo rats (*Dipodomys*) and meadow voles (*Microtus*), males have larger home-ranges, better spatial learning ability, and a larger hippocampus than more sedentary females (Jacobs and Spencer 1994; Jacobs et al. 1990). In the teleost fishes, the dorsolateral region (Dl) of the telencephalon is involved in spatial learning (Salas et al. 1996a, b; Vargas et al. 2000, 2009) and is homologous to the hippocampus (Rodríguez et al. 2002; Broglio et al. 2003; Northcutt 2006). Like the hippocampus, larger Dl size in the telencephalon is associated with larger home range sizes (Carneiro et al. 2001) and residence in structurally complex littoral habitat (Shumway 2008). In a broad comparison of the shape of the telencephalon in the benthic and limnetic threespine stickleback which included populations used in this study (Park and Bell 2010), the dorsolateral region of the telencephalon tended to be more convex, and thus, probably more voluminous, in benthic than in limnetic

populations. Thus, greater size of the dorsolateral region of the telencephalon may be associated with superior spatial learning in the threespine stickleback.

Boldness in threespine stickleback is more prevalent in lake populations with native fish predators than those without them (Huntingford et al. 1994). Because all lake samples in the current study came from habitats with native predatory fishes, population differences for boldness were not expected. Bolder individuals are more likely to explore novel surroundings (Budaev 1997), possibly leading to faster spatial learning than in shy individuals (see Burns and Rodd 2008). Shy individuals exhibit more freezing behavior than bolder individuals (Walsh and Cummins 1976; Templeton and Shriner 2004; Burns and Rodd 2008). The relative number of freezes during the time interval between first leaving the starting area and entering a room in an experiment's first trial was taken as a measure of boldness for each fish, with lower values indicating bolder behavior. Consistent with expectations, ecotypic differences for boldness were not found in the present study.

During trial 1, the propensity for exploratory behavior should be proportional to the amount of ambulation by a fish before it entered a room for the first time. Compared to limnetics, benthics may be expected to exhibit more exploratory behavior because they reside in lakes with more structurally complex habitats. In birds and mammals, the size of the hippocampus, and therefore, presumably spatial learning, is associated with residence in structurally complex habitats (Sherry 1998). However, unlike spatial learning, no differences in exploratory behavior were detected between Alaskan threespine stickleback ecotypes. Latency to emerge from a starting area in a novel environment can be treated as a trade-off between shyness and willingness to explore (Burns 2008). In the present study, it was recorded as the time required to leave the starting area during each subject's first exposure to the T-maze (i.e., trial 1).

Ecotype did not influence latency to emerge as well as more direct measures of boldness and exploratory behavior, which suggests that Alaskan threespine stickleback were more-or-less similar in their response to introduction to the T-maze.

The anadromous Rabbit Slough stickleback population in Experiment 2 was used to infer the ancestral state of spatial learning ability (see Bell 1995; Park and Bell 2010). Since the resident lake populations sampled exhibit spatial learning, it was very likely that variation for spatial learning also existed in the anadromous ancestor. To examine the influence of experience on spatial learning in the sea-run population, fish were reared in spatially contrasting aquaria. Fish reared in the spatially complex aquarium were expected to exhibit superior spatial learning than those reared in the spatially simple aquarium. Contrary to this expectation, rearing treatment did not influence spatial learning ability significantly. None of the performance variables measured during trial 1 or during criterion trials differed between rearing treatments.

There are three possible explanations for the negative findings between fish from Rabbit Slough reared in the spatially contrasting aquaria. First, experience may not be important for the expression of spatial learning. Alternatively, the influence of experience may be substantial, but the artificial spatially complex rearing treatment may not have been complex enough to elicit phenotypic plasticity for spatial learning ability. Finally, the rearing treatments may have affected spatial learning but sample sizes were too small to detect differences. Environmental enrichment induces neurological changes associated with enhanced spatial learning (Juraska et al. 1985, 1989; Patel et al. 1997; van Praag et al. 2000; Rampon et al. 2000; Vargas et al. 2000; Olson et al. 2006). Girvan and Braithwaite (1998) detected learning differences using field-caught pond and river stickleback populations, but when they replicated this comparison using lab-reared fish from spatially contrasting aquaria, these differences disappeared (Girvan and

Braithwaite 2000). These results and those from Experiment 3 (see below) suggest that experience is important for spatial learning and that the artificial spatial complexity used in Experiment 2 may have been insufficient to induce differences in spatial learning ability. Further study using larger sample sizes and more complex rearing environments may reveal an effect of experience in threespine stickleback and provide general insight into whether the acquisition of necessary spatial experiences occurs gradually or during critical periods (see Thorpe 1958; Penfield and Roberts 1959; Almli and Finger 1987).

Despite the absence of an overall rearing effect in Experiment 2, a substantial number of Rabbit Slough fish from both rearing treatments still solved the maze (Table 3). This strongly suggests that ancestral anadromous threespine stickleback possess a substantial genetic potential for spatial learning. They may use spatial learning for behaviors other than foraging, such as migration (Fukumori et al. 2010), predator evasion (Aronson 1951, 1971; Markel 1994; Burns and Rodd 2008), or territoriality during the breeding season (Lamanna and Eason 2003). West-Eberhard's (2001) developmental-plasticity hypothesis proposes that derived traits of descendant populations are the products of sorting of ancestral variation, such as life stages or alternative phenotypes. The present results taken with the earlier findings of Park and Bell (2010) support the following hypothesis: because brain tissue is metabolically expensive to maintain (Hawkins 1985; Metcalf & Ure 1995; Dukas 1999), reduced advantages of spatial learning in non-migratory, limnetic stickleback in lakes may have favored reduction in the size of the dorsolateral region of the telencephalon.

Experiment 3 explored the contribution of experience to spatial learning in a freshwater benthic population. Lab-reared Corcoran Lake fish were compared to their field-caught counterparts in Experiment 1 because results from Experiment 2 and from Girvan and

Braithwaite (2000) suggest that artificial complexity in an aquarium may not affect spatial learning ability. Field-caught Corcoran Lake fish took fewer trials to reach the criterion than lab-reared fish, and fewer lab-reared fish solved the maze. These results are consistent with the hypothesis that spatial learning is strongly dependent on prior experience with spatial complexity. Welfare differences between field-caught and lab-reared samples were unlikely to account for the differences in spatial learning. Although treatment of pathogens in field-caught fish may be expected to hinder their performance during an experiment, it was the untreated lab-reared Corcoran Lake fish that performed poorly. However, a possible alternative explanation for the results in Experiment 3 could be differential mortality in field-caught fish. Unlike lab-reared fish which were a random sample from a mass cross, field-caught fish may have been superior learners because poorer learners do not survive in the wild. Unfortunately, the current methods cannot rule out this explanation. Thus, the relationship between experience and spatial learning remains unclear in threespine stickleback, but the present results strongly suggest that this species has enormous potential for further investigations into the behavioral ecology, plasticity, and evolution of cognition.

In Experiment 3, lab-reared fish were bolder than field-caught fish, but all remaining performance variables did not differ between samples. Again, bolder individuals are more likely to explore a novel environment (see Budaev 1997), which could bias them to acquire spatial learning sooner than shy individuals (see Burns and Rodd 2008). Contrary to this expectation, lab-reared fish did not exhibit a greater tendency to explore or ability to solve the maze. This was a conservative difference because lab reared fish, despite being bolder by this measure, learned less well. One possible explanation for the difference in boldness is that fish reared in the lab were more experienced to artificial surroundings and handling by observers, and therefore, they

exhibited less shy behavior than their field-caught counterparts. Another possibility could be that field-caught fish had been chased by their fathers while they were beginning to stray from the nest or by predators during the year before they were collected. Finally, again it could be due to differential mortality. Stickleback from lakes with a greater variety of fish predators exhibit more shy behavior (Huntingford et al. 1994). Therefore unlike lab-reared fish, it is possible that the field-caught fish that were sampled included a greater number of shy individuals or generally shyer individuals because the bolder ones are typically selected against (see Brown and Braithwaite 2004). Further study may reveal whether the difference in boldness between lab-reared and field-caught fish from this population was due to experience or selective mortality prior to capture.

In threespine stickleback, benthics occupy littoral environments, have rounder telencephalon shapes (Park and Bell 2010), and are superior spatial learners than limnetics (Odling-Smee et al. 2008; this study). Greater telencephalon convexity may reflect greater volume of Dl in the telencephalon, and Dl is involved in spatial learning in fishes. In ninespine stickleback (*Pungitius pungitius*), laboratory-reared marine populations have larger telencephala than pond populations (Gonda et al. 2009), indicating that telencephalon differences for *Pungitius* populations are heritable. Thus, there are likely to be similar differences in spatial learning among *Pungitius* populations that could be related to environment.

Vertebrates can use an array of learning strategies to solve spatial tasks (Nadel 1994; Schacter and Tulving 1994; López et al. 1999), but most previous findings are based on birds and mammals (Dodson 1988; Healy 1998). The current study investigated the evolution of spatial learning to the exclusion of cue learning or turn discrimination in threespine stickleback. Unlike the latter learning strategies, spatial learning takes into account the processing of an array of

visual landmarks. Benthics exhibited superior spatial learning to that of limnetics, and in at least one benthic population, spatial learning may depend considerably on phenotypic plasticity. Furthermore, anadromous stickleback exhibit spatial learning, supporting West-Eberhard's (2003) developmental-plasticity hypothesis. Multiple components of learning (e.g., motivation, reinforcement, discernment, memory) may be affected during evolution of spatial learning (see West-Eberhard 2003), and future work on fishes should aim to identify all these components, constraints among them, and their relationship to the dorsolateral region of the telencephalon (see Warburton 2003).

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Table 1. Criteria for ecotype classification of study populations. The relative littoral area of lakes (RLA) is the area of the lake bottom deeper than the isobath for euphotic zone depth divided by lake surface area. Gill raker number, stomach contents, dental microwear, lateral body and skull shape, and foraging behavior were used to classify ecotypes. Sources of information on lake or stickleback properties are described in the materials and methods. Unavailable data are marked with hyphens. Table modified from Park and Bell (2010).

Population	Region	RLA[2]	Gill Rakers[2]	Stomach Contents[5]	Microwear[5]	Body[6]	Skull[7]	Foraging[8,9]
Long	Mat-Su	Limnetic (30%)	Limnetic (21.9)	(32.6% B, 67.4% L)	Limnetic	Limnetic	Limnetic	Limnetic
Lynne	Mat-Su	Limnetic (51%)[1]	Limnetic (21.1)[4]	–	–	–	Limnetic	Limnetic
Matanuska	Mat-Su	Limnetic (10%)[1]	–	–	–	Limnetic	Intermediate	Limnetic
South Rolly	Mat-Su	–	Limnetic (21.4)[4]	–	–	Limnetic	Limnetic	Limnetic
Corcoran	Mat-Su	Benthic (100%)	Uncertain (20.5)	(79.2% B, 20.8% L)	Benthic	Benthic	Benthic	–
Mud	Mat-Su	Benthic (100%)	Benthic (18.0)	(93.4% B, 6.6% L)	Benthic	Benthic	Benthic	–
Willow	Mat-Su	Benthic (100%)[1]	Benthic (19.9)[3]	(57.2% B, 42.8% L)[3]	Benthic	Benthic	Intermediate	–
Walby	Mat-Su	Benthic (78%)[1]	Benthic (19.8)[4]	–	–	Benthic	Benthic	–
Stormy	Kenai	Limnetic (33%)	Uncertain (20.4)	–	–	Limnetic	Intermediate	Limnetic
Tern	Kenai	Benthic (100%)[1]	Benthic (17.7)[4]	–	–	Benthic	Benthic	–
Rabbit Slough	Mat-Su	–	Limnetic (22.4)[3]	(72.8%B, 27.2%L)[3]	–	–	–	–

[1]M.A. Bell (unpublished data) or [2]Walker (1997)
[2]Walker (1997), [3]M.P. Travis (unpublished data), or [4]Park (unpublished data)
[3]M.P. Travis (unpublished data) or [5]Purnell *et al.* (2006)
[5]Purnell et al. (2006)
[6]W.E. Aguirre (unpublished data)
[7]Willacker et al. (2010)
[8]Foster (personal communication 2006) and [9]Baker (personal communication 2008).

Table 2. Behavioral variables for spatial learning experiments and their descriptions. Boldness, exploratory behavior, and latency to emerge were measured only during the first trial of experiments. "Criterion" values refer to the results from the six successful criterion trials. Transformations (Transform.) used to normalize residuals of data used in ANOVA tests are given (N/A, not applicable). The categories of subjects used in statistical analyses are as follows: subjects that solved the maze, S; that did not solve the maze, NS; that were encouraged ten times before reaching the maximal 50 trials, E.

Variable Name (Y)	**Measure**	**Transform.**	**Subjects**	**Trial(s)**	**Test for**
Trials to reach criterion	Trials to reach criterion	N/A	S + NS + E	N/A	Spatial learning
No. of fish (not) reaching criterion	No. of fish (not) reaching criterion	N/A	S + NS + E	N/A	Spatial learning
Boldness	No. of freezes	√(Y)	S + NS + E	1	Bold or shy behavior
Exploratory behavior	Novel ambulation	√(Y)	S + NS + E	1	Tendency to explore
Latency to emerge	Novel latency to emerge	LOG(Y)	S + NS + E	1	Shyness-exploration conflict
Activity level	Experienced ambulation	√(Y)	S	Criterion	Active or passive behavior
Starting time	Experienced latency to emerge	LOG(Y)	S	Criterion	Time leaving starting area
Food time	Time to complete trial	LOG(Y)	S	Criterion	Time to reach food reward

Table 3. Results for spatial learning experiments. The experiment number is shown on the left above corresponding samples. Three categories of results are shown: fish that reached a criterion of six out of seven trials without a wrong-door error (S), fish that could not solve the maze in 50 trials (NS), fish that were terminated early because they were "encouraged" ten times (E), and fish lost due to attrition (A). Year of experiment (Year) is listed on right.

Exp/Sample	**Ecotype**	**S**	**NS**	**E**	**A**	**Year**
Experiment 1:						
Long	Limnetic	10	3	3	0	2006
Lynne	Limnetic	7	3	6	0	2006
Matanuska	Limnetic	8	7	1	0	2006
South Rolly	Limnetic	2	0	9	0	2008
Stormy	Limnetic	3	0	6	2	2008
Corcoran*	Benthic	10	5	1	0	2006
Mud	Benthic	5	1	5	0	2008
Walby	Benthic	11	4	1	0	2006
Willow	Benthic	10	6	0	0	2006
Tern	Benthic	2	1	5	3	2008

Experiment 2:

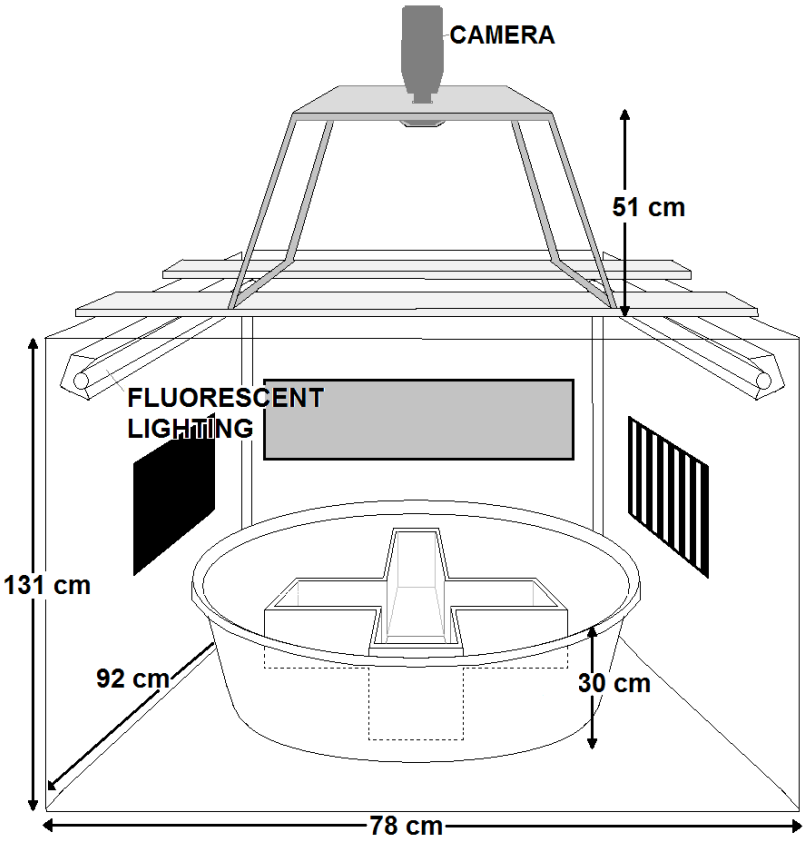
Rabbit Slough (complex)	Sea-Run	8	1	4	0	2007
Rabbit Slough (simple)	Sea-Run	5	1	7	0	2007

Experiment 3:

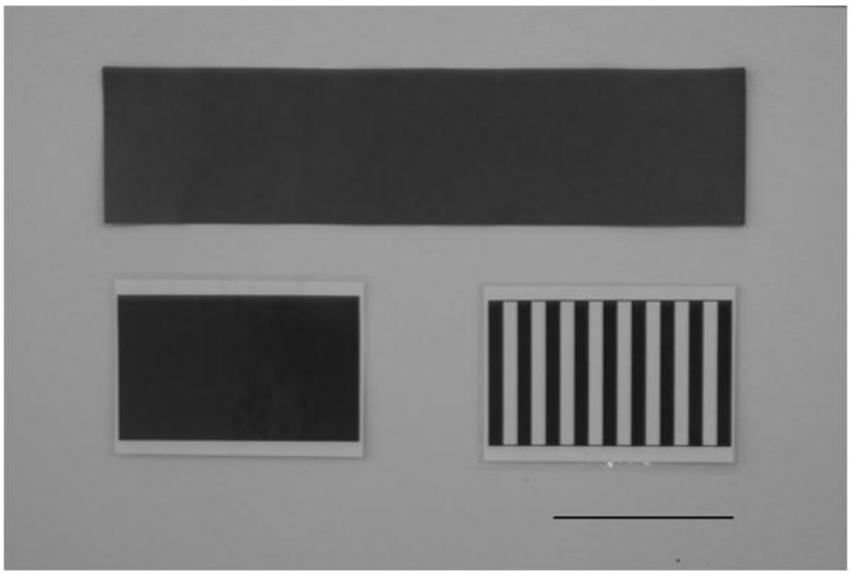
Corcoran (lab-reared)	Benthic	3	8	7	0	2008
Corcoran* (field-caught)	Benthic	10	5	1	0	2006

**Same dataset.*

Figure 1. Experimental enclosure and extra-maze cues. (a) Experimental enclosure, camera stand, extra-maze cues, and pool with four-arm maze (not to scale). See text for dimensions of four-arm maze. (b) The three extra-maze visual cues used for all experiments. Scale bar is 20 cm.



(a)



(b)

Figure 2. Schematic outline of Experiments 1-3 and T-maze protocol for testing spatial learning. (a) Experiment 1. Ecotypic differences in spatial learning in field-caught samples. (b) Experiment 2. Influence of experience in lab-reared fish from an anadromous ancestral population reared in spatially contrasting aquaria. (c) Experiment 3. Influence of experience on spatial learning using lab-reared and field-caught benthic fish from Corcoran Lake. (d) The T-maze protocol (center) required the randomization of the starting area (SA) between positions 180° apart; a fish assigned food to the left side of the enclosure entrance (▲) is shown. To facilitate spatial learning, three extra-maze cues (solid black short, banded black-and-white short, and solid blue elongate boxes) were placed on the walls within the experimental enclosure. The spatial relationship of the food reward (F) to the extra-maze cues remained unchanged for each fish through trials. Not drawn to scale.

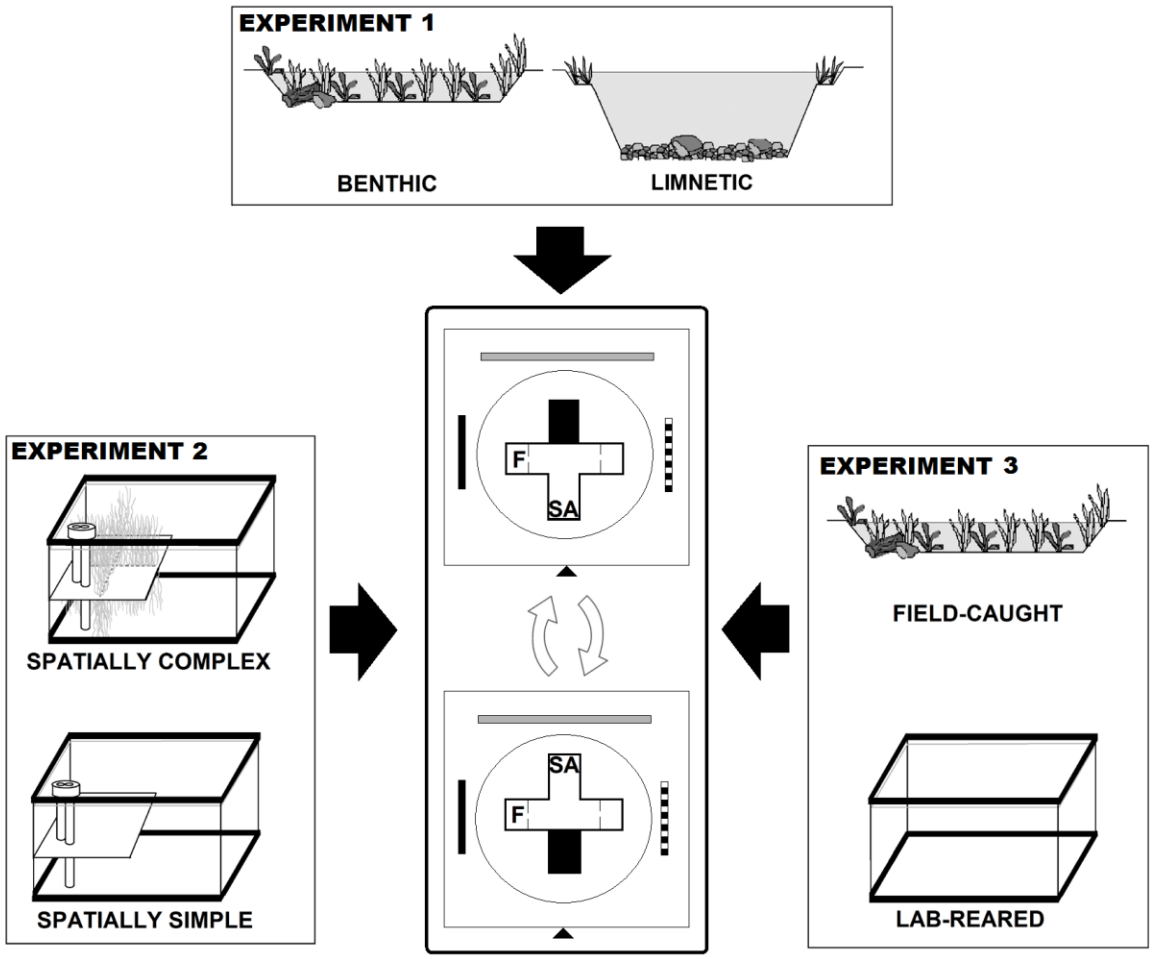


Figure 3. Spatial learning in field-caught samples (Experiment 1). Mean values and standard errors of cumulative trials to reach the criterion of correctly choosing the food room in six out of seven trials shown for field-caught samples of benthic and limnetic populations. Individuals lost due to attrition (see Table 3) are not included.

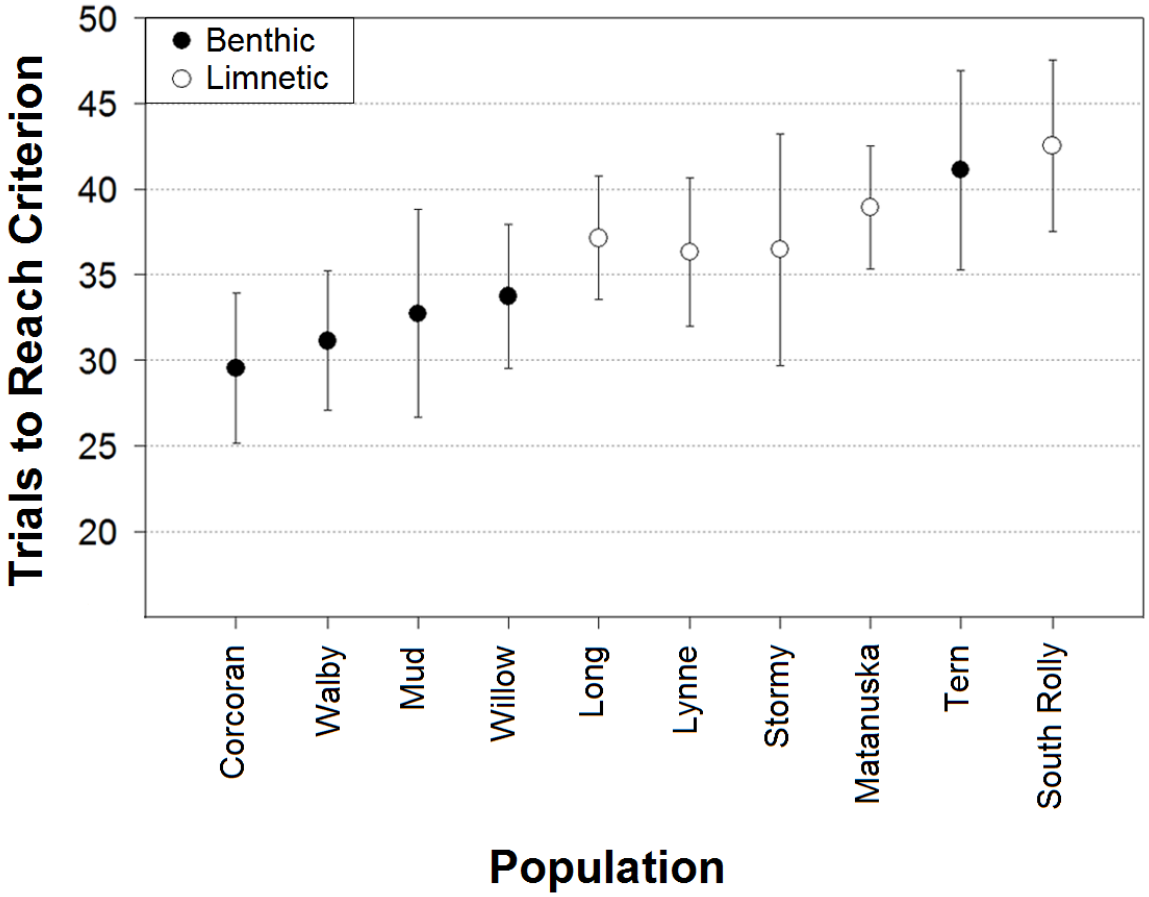


Figure 4. Evaluation of the six out of seven criteria. The relative proportion of correct choices made by fish that achieved the criterion of correctly choosing the food room in six out of seven trials was plotted for the first 20 trials. The proportion of correct choices made by subjects increased with trial number, indicating that fish were learning to avoid the unrewarded room as trials progressed.

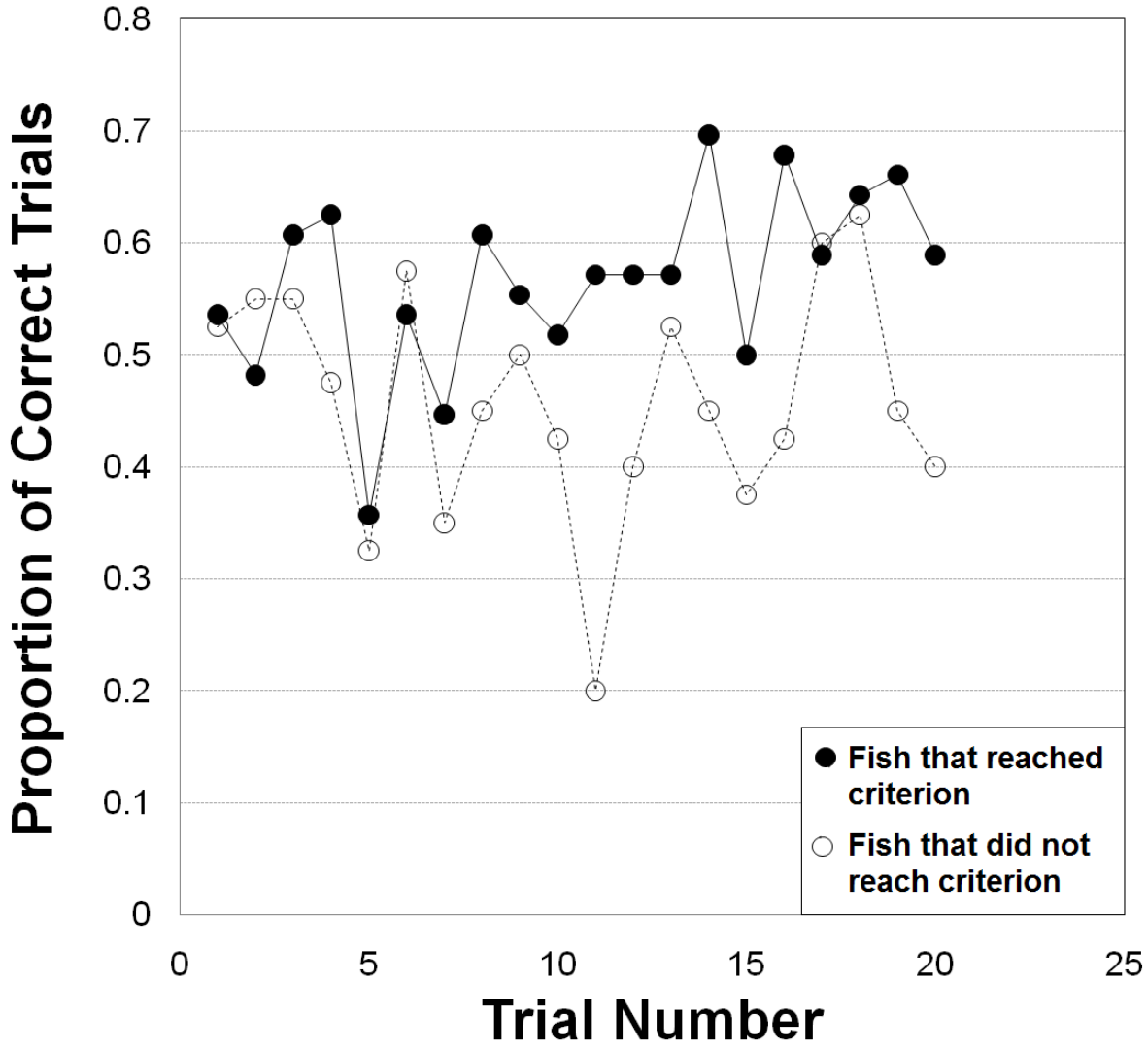


Figure 5. Spatial learning differences in lab-reared and field-caught Corcoran Lake fish (Experiment 3). Mean values and standard errors of cumulative trials to reach the criterion of correctly choosing the food room in six out of seven trials shown for all fish from field-caught and lab-reared benthic fish from Corcoran Lake.

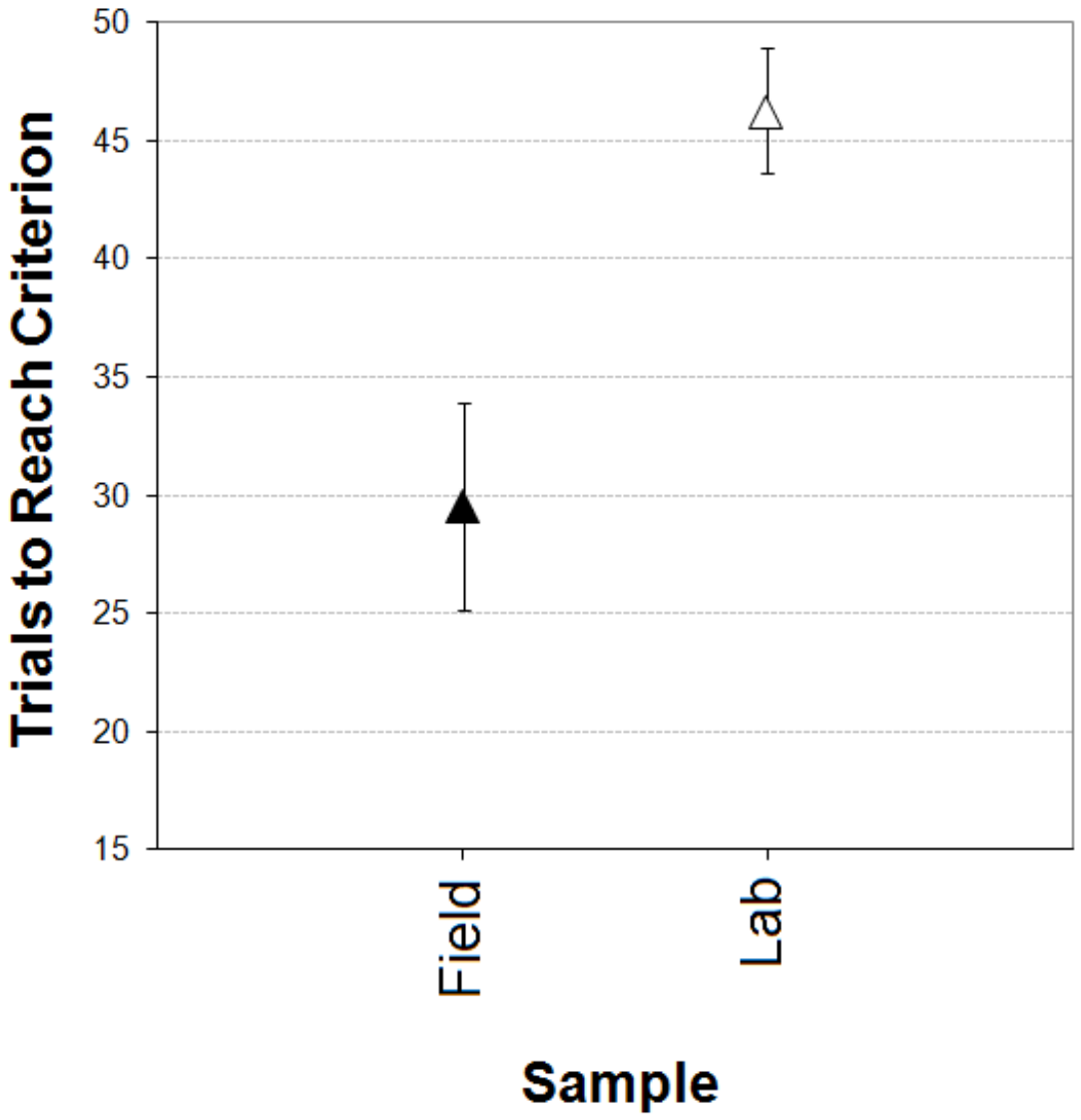
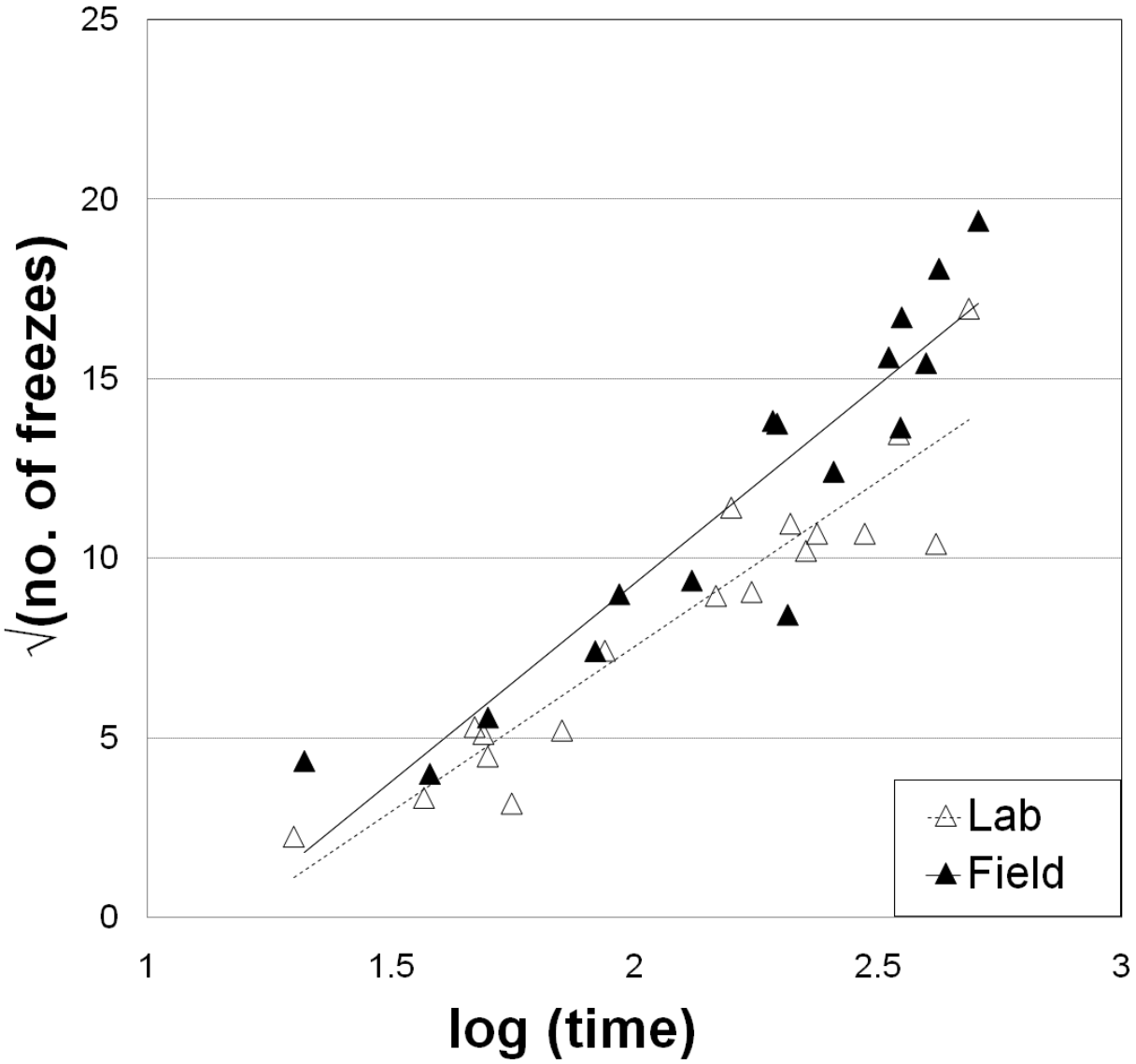


Figure 6. Boldness in lab-reared Corcoran Lake fish (Experiment 3). To compare boldness between lab-reared and field-caught Corcoran Lake samples, square root-transformed total number of freezes was regressed on the log of the time interval between when a fish left the starting area and first entered a room during trial 1. An ANCOVA detected a significant difference between samples, with lab-reared fish exhibiting more boldness.



Chapter 3

Variation of Telencephalon Morphology of the Threespine Stickleback (*Gasterosteus aculeatus*) in Relation to Inferred Ecology

ABSTRACT

We tested the hypothesis that increased telencephalon size has evolved in threespine stickleback fish (*Gasterosteus aculeatus*) from structurally complex habitats using field-caught samples from one sea-run (ancestral) and 18 ecologically diverse freshwater (descendant) populations. Freshwater habitats ranged from shallow, structurally complex lakes with benthic-foraging stickleback (benthics), to deeper, structurally simple lakes in which stickleback depend more heavily on plankton for prey (generalists). Contrary to our expectations, benthics had smaller telencephala than generalists, but the shape of the telencephalon of the sea-run and benthic populations were more convex laterally. Convex telencephalon shape may indicate enlargement of the dorsolateral region, which is homologous with the tetrapod hippocampus. Telencephalon morphology is also sexually dimorphic, with larger, less convex telencephala in males. Freshwater stickleback from structurally complex habitats have retained the ancestral telencephalon morphology, but populations that feed more in open habitats on plankton have evolved larger, laterally concave telencephala.

INTRODUCTION

Understanding evolution of the vertebrate telencephalon is crucial to link behavioral ecology with neurobiology. Due to the high metabolic cost of brain tissue, brain morphology should reflect functional importance for complex behaviors (Dukas, 1999). The Clever Foraging Hypothesis (CFH) states that active predators, unlike grazers, have evolved larger brains because greater neurobiological capability is needed to search "strategically" for their food (Parker & Gibson, 1977; Striedter, 2005). The CFH has been proposed to explain the evolution of complex brain morphology and behavior (Parker & Gibson, 1977; Striedter, 2005). In fishes, the relationship between brain size and diet or habitat has been studied in sharks (Yopak *et al.*, 2007) and cichlids (Gonzalez-Voyer *et al.*, 2009; Huber *et al.*, 1997; Pollen *et al.*, 2007) and was reviewed by Kotrschal *et al*. (1998) and Ito *et al*. (2007). However, only a few studies (see Burns & Rodd, 2008; Burns *et al.*, 2009; Gonda *et al.* 2009) have considered differences in telencephalon morphology among ecologically contrasting conspecific populations of fishes. We used field-caught samples from Alaskan populations of threespine stickleback (*Gasterosteus aculeatus*) to study the relevance of the CFH to telencephalon morphology on a well-known adaptive radiation in fishes.

The CFH emphasizes the importance of foraging ecology in driving neuroanatomical change, and its predictions have been confirmed in mammals (see Joliceur *et al.*, 1984; Gittleman, 1986; Hutcheon *et al.*, 2002; Striedter, 2005) and birds (see Healy *et al.*, 2005). In bats, insectivores rely predominantly on echolocation to catch insect prey while frugivores, which have larger brains, must locate and time the ripeness of fruits (Eisenberg & Wilson, 1978; Joliceur *et al.*, 1984; Hutcheon *et al.*, 2002). In primates (Clutton-Brock & Harvey, 1980) and small mammals (Mace *et al.*, 1981), insectivores and frugivores have larger relative brain size

than grazers. In the Carnivora, larger brain size is associated with species that have complicated foraging strategies (Gittleman, 1986). Furthermore, unlike simpler learning strategies that lack neuroanatomical correlates, spatial learning during foraging is associated with a larger hippocampus in the forebrain of tetrapods (O'Keefe & Nadel, 1978; Sherry, 1998; Healy *et al.*, 2005).

The gross morphology of teleost brain lobes provides quantifiable measures of how a species has adapted to its environment (Davis & Miller, 1967; Kotrschal *et al.*, 1998). The forebrain is involved in numerous functions, including classical conditioning (Flood *et al.*, 1976; Overmeir & Hollis, 1983), processing sensory information (Davis & Kassel, 1983; Hofmann, 2001), aggression (Davis & Kassel, 1983), solving complex social tasks (Huber *et al.*, 1997; Kotrschal *et al.*, 1998; Hofmann, 2001), mating and reproduction (Demski & Beaver, 2001), schooling (Davis & Kassel, 1983; Shinozuka & Watanabe, 2004), avoidance learning (Portavella *et al.*, 2003, 2004; Vargas *et al.*, 2009), and spatial learning (Broglio *et al.*, 2003; Vargas *et al.*, 2000; Vargas *et al.*, 2009). Teleosts that forage in structurally complex Hawaiian reefs have larger telencephala than sedentary species (Bauchot *et al.*, 1977), supporting the CFH. The telencephalon is also larger in African cichlid species that forage in complex, benthic microhabitats than in those from pelagic microhabitats (van Staaden *et al.*, 1994/95; Huber *et al.*, 1997; Pollen *et al.*, 2007).

The threespine stickleback (*Gasterosteus aculeatus*) is an excellent species in which to test the CFH. Like other teleosts, its brain consists of a pair of olfactory bulbs, a pair of forebrain, optic, and hypothalamus lobes, and a single cerebellum (Nieuwenhuys, 1959, 1962; Segaar & Nieuwenhuys, 1963; Ekström & van Veen, 1983, 1984; Ekström *et al.*, 1986; Ekström *et al.*, 1990; Fig. 1A, B). The teleost forebrain is divided into the telencephalon and

diencephalon. The telencephalon can be further subdivided into three pallial regions dorsally and two subpallial regions ventrally. Pallial regions are generally considered homologous across teleost species, but determining homologies among nuclei within each region remains challenging (Nieuwenhuys *et al*., 1998b).

There are three major forms of threespine stickleback, marine, sea-run, and freshwater, and the sea-run form has founded innumerable freshwater populations (e.g., Bell & Foster, 1994; Bell, 1995). Sea-run threespine stickleback populations are distributed world-wide in temperate coastal environments and typically exhibit limited variation for armor traits (Colosimo *et al.*, 2005) and body shape (Walker & Bell, 2000). They feed on plankton at sea (Mackney & Hughes, 1995) and have trophic morphology resembling limnetics (McPhail, 1994). Adaptation to a wide range of freshwater habitats has generated highly divergent morphological phenotypes among lake populations (reviewed in Bell & Foster, 1994; Reimchen, 1994; McPhail, 1994; Walker, 1997). Threespine stickleback from shallow, densely vegetated lakes (i.e., benthics) forage mostly on benthic invertebrates (Schluter & McPhail, 1992; Hart & Gill, 1994). In contrast, plankton specialists (i.e. limnetics) eat plankton in open water. Compared to limnetics, benthics generally have a deep head and body, smaller eyes, and fewer, shorter gill rakers (reviewed in Bell & Foster, 1994; McPhail, 1994; Walker, 1997).

According to the CFH, foraging on benthos should be associated with larger telencephalon size among threespine stickleback populations. However, unlike shallow lakes that include exclusively benthic habitat, deep lakes include both littoral habitat near shore and limnetic habitat off shore. Therefore, like benthics, limnetics may rely on spatial information within benthic microhabitats, especially during the breeding season, when mating occurs near shore. We sampled lake populations from the Matanuska-Susitna Valley (n=15) and Kenai

Peninsula (n=3), Alaska. Each lake sample was collected once within daylight hours during the breeding season (late May - mid-June). Other sample properties are summarized in Table 2. All populations were classified as benthic or relatively limnetic based on a series of indirect measurements, and telencephalon size and shape were analyzed. However, stomach content analysis (M. Travis, unpublished data) revealed that many putatively limnetic populations, based on indirect evidence, consume both plankton and benthic prey. Therefore, we refer to them as "generalists." Based on existing information on teleost fishes and without prior knowledge of ecotypic telencephalon differences in stickleback, we hypothesized that benthics rely relatively more on spatial information, and therefore, should have larger relative telencephalon sizes than generalists.

We used the telencephalon morphology of one sea-run stickleback population from the Matanuska-Susitna Valley to indicate the ancestral condition for the derived lake populations (see Bell, 1995). Dramatic contemporary evolution (e.g., Bell *et al.*, 2004) and diversity among freshwater populations of threespine stickleback for other traits (reviewed in Bell & Foster, 1994; Reimchen, 1994; McPhail, 1994; Walker, 1997) suggest that telencephalon morphology may also have undergone substantial diversification in the derived lake populations within the last 22,000 years, the maximum age of our study populations (Reger & Pinney, 1996).

We also studied telencephalon sexual dimorphism. During the breeding season, reproductive male stickleback attempt to establish a mating territory, within which they build nests that they defend against conspecifics and predators and conduct most of their foraging and parental behavior (e.g., Wootton, 1976; Rowland, 1994; Foster, 1994a; Mori, 1998; Candolin & Voigt, 2001). Male site fidelity during parental care may contribute to enlarged forebrain size. Males also provide sole care for offspring, a behavioral trait associated with enlarged brains in

cichlids (Gonzales-Voyer *et al.*, 2009). In contrast, females exhibit less parental care and site fidelity, often ranging widely to forage and engage in courtship. However, female stickleback may evaluate different male territories before choosing to mate, which may involve utilizing spatial information from the male territory. Larger homerange size is associated with greater hippocampus size in tetrapods (see Sherry, 1998) and greater telencephalic dorsolateral region size in teleosts (Carneiro *et al.*, 2001). However, it is not clear whether female stickleback maintain a homerange or rely on individual visual landmarks during mate choice. Although both sexes may utilize spatial information, they use it differently, and we suspect that males exhibit more site fidelity than females. Thus, we expected males to be more dependent on information on habitat complexity and parental care and therefore to have larger telencephala.

MATERIALS AND METHODS

Field Samples

Specimens of *G. aculeatus* were collected from freshwater sites near Cook Inlet, Alaska using unbaited wire mesh G minnow traps (chamber 44.5 cm long, 22.9 cm diameter; mesh 0.32 or 0.64 cm) set for roughly 24 h in heterogeneous microhabitats at < 1 m depth near shore. Locations and ecological information for the samples came from Bell and Ortí (1994), an unpublished database, or public records (Sport Fish Division, Alaska Department Fish and Game, http://www.adfg.state.ak.us/). Locations and sample properties are summarized in Table 2. All specimens were sacrificed by overdose with MS-222. Specimens from 12 populations were fixed in 10% formalin in 0.1 M (10.9 g dibasic: 2.5 g monobasic: 1 L deionized water) sodium phosphate buffer solution (PBS), which is isotonic with fish tissue and buffered at pH 7.4 (method A). Another seven samples were fixed in 10% formalin buffered with calcium carbonate

(dolomite) in lake water because they were originally preserved for other purposes (method B). These fixation protocols do not appear to affect neuromorphology significantly because ecologically similar populations fixed by methods A and B produced comparable results. Sampling was approved by the Alaska Department of Fish and Game and by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University (method A) or Clark University (method B).

Stickleback samples came from nine putatively generalist populations, seven putatively benthic populations, one stream, one shallow lake with apparently limnetic fish (Kashwitna Lake), and one sea-run population (Table 1) from the Matanuska-Susitna Borough of Cook Inlet, Alaska. Additional populations from Engineer, Stormy, and Tern lake samples come from the Kenai Peninsula, Alaska. All sixteen lakes contain native predatory fishes. Where available, relative littoral area, gill raker number, body shape, stomach contents, dental microwear, and foraging behavior data were used to infer diet (Table 2). Relative littoral area is the proportion of a lake that can support rooted plants. We used water transparency (M.A. Bell, unpublished data) to estimate euphotic zone depth (Koenings *et al.*, 1987), and the isobath for euphotic zone depth was interpolated on published (http://www.adfg.state.ak.us/) and unpublished bathymetric maps provided by the Sport Fish Division of the Alaska Department of Fish and Game. We estimated relative littoral area by digitizing the area within the isobath for euphotic zone depth and within the lake shore, subtracting the former from the latter, and dividing the difference by lake surface area (see Walker, 1997). Higher values for relative littoral area indicate greater structurally complex benthic habitat, which should be associated with benthic diet and trophic morphology.

Ecologically similar populations from different drainages are unlikely to share non-ancestral (i.e., derived) neuromorphology due to common ancestry (Bell, 1995). Some statistical

comparisons were limited to a subset of ten populations, selected so that no two of them with the same putative ecotype came from the same drainage (Table 2). To increase the chances of detecting ecotypic differences in telencephalon morphology, where available, we intentionally chose populations with the most extreme differences in telencephalon shape, body shape, diet, dental microwear, and foraging behavior. Thus, neuroanatomical similarities within the sets of benthic and generalist populations were free to evolve independently, and all ten populations can be treated potentially as independent samples. However, if populations with the same ecotype have retained ancestral (i.e., sea-run) traits, then phenotypic similarities between lake and sea-run populations may be due to common ancestry and not evolutionary convergence. Therefore, we refer to the ten samples in these additional analyses as "potentially independent."

Sex was determined by examination of the gonad through an incision on the right side posterior to the pelvis and inspection under a dissecting microscope. The testis is usually opaque and melanized, while the ovary is larger with a translucent, unmelanized membrane encasing the ova, which may be visible through its wall.

Preparation and Imaging of Brains

Prior to brain extraction, each specimen was rinsed in distilled water and soaked in 0.1 M PBS for 3 h to dilute the formalin. Standard length (SL, distance from the tip of the upper jaw to the end of the vertebral column) and the anterio-posterior diameter of the left eye were measured using digital calipers. Specimens were immediately placed under a dissecting microscope, and their brains were extracted dorsally after removing the parietal, frontal, and nasal bones, and in some cases, the right operculum. Fine forceps were used to sever the cranial nerves and the posterior end of the hindbrain. Applying pressure with forceps to the ventral portion of the brain

case dislodged the brain without severing the pituitary gland. The brain was dipped periodically throughout this procedure in 0.1 M PBS buffer to prevent desiccation. Each brain was stored in a 1.5 ml tube with 10% formalin in 0.1 M PBS.

Digital images of the dorsal aspect of the brain were taken using a computer-assisted video image analysis system, Sony DXC-390 (SONY, Minato, Tokyo, Japan) video camera, mounted on a Leica MZ75 microscope. Images were acquired using ImagePro software (version 4.1.0.0 for Windows 95/NT/98, Media Cybernetics, Silver Spring, MD, USA, 1999). Magnification on the microscope was always set to 6.3X, and images were saved in TIF format.

Geometric Morphometrics

Geometric morphometric methods (Rohlf & Marcus, 1993; Zelditch *et al.*, 2004) were used to study telencephalon shape variation using only the left lobe (Fig. 1C). The dorsal aspect of the telencephalon has few discrete landmarks along its lateral edge. Therefore, only a pair of extreme landmarks (i.e., LM 1, LM 14) was fixed to defined points, and 12 intervening semi-landmarks (Bookstein, 1991; Bookstein, 1996/97; see below) were digitized along the lateral edge of the telencephalon lobe at roughly even intervals using tpsDig version 2.05 (Rohlf, 2006). From the dorsal aspect, the posterior part of the forebrain abuts the midbrain-tegmentum. We do not use the term "optic lobe" here because our methods cannot distinguish the optic tectum from other midbrain parts. The fourteen landmarks were placed successively at the following points (Fig. 1C): LM 1, posteromedian juncture of the right and left telencephalon lobes (fixed); LM 2, most posteromedian edge of telencephalon where it abuts the left midbrain-tegmentum; LM 3, median edge of sulcus ypsiloniformis; LM 4, lateral edge of sulcus ypsiloniformis; LM 5, midpoint between LM 4 and 6; LM 6, most posterolateral edge of telencephalon where it abuts

the midbrain-tegmentum; LM 7 to LM 11 were at equal intervals between LM6 and LM 12; LM 12, most anterolateral edge of telencephalon where it abuts olfactory lobe; LM 13, anteromedian edge of telencephalon where it abuts the olfactory lobe; LM 14, anteromedian juncture of the right and left telencephalon lobes where they abut the olfactory bulbs (fixed).

Using a slider's file, the semi-landmark data were "slid" and aligned using Procrustes superimposition, as implemented in tpsRelw version 1.42 (Rohlf, 2005), to eliminate variation from rotation, translation, and size. The semi-landmark technique allows superimposition of shapes that do not have many obvious landmarks that can be accurately digitized, but it requires that the first (LM 1) and last (LM 14) landmarks remain fixed (Bookstein, 1996/97). Each intermediate landmark can "slide" along a line parallel to a line connecting the semi-sliding landmark to the landmark's immediate left and right. All specimens (Table 2) were included in a single alignment from which the shape variables (i.e., partial warps, uniform component) were generated.

As a check of the shape alignment using all samples, a separate alignment was done for the subset of ten potentially independent lake samples. In this subset of ten samples, an additional standardizing variable, brain length (BL, distance along the dorsal mid-line from the anterior telencephalon landmark, LM 14 in Fig. 1C, to the posterior end of the cerebellum), was measured using these images, and TpsRegr 1.31 (Rohlf, 2005) was used to acquire telencephalon centroid sizes from the landmark data. Centroid size is the square-root of the sum of squared distances of each landmark from the midpoint of all 14 landmarks. Unlike a single linear measure, it takes distances in multiple directions into account.

Please see Appendix 2 for analyses validating the use of left telencephalon centroid size as a proxy for telencephalon volume estimated from histological sections and that the time brains were stored in formalin between brain extraction and image capture did not affect results.

Multivariate Analysis

TpsRelw version 1.42 (Rohlf, 2005) was used to perform a Principal Components Analysis (PCA) of the landmark data. PCA summarizes shape variation into a low-dimensional morphospace representing the major axes of shape variation. A PCA was carried out on the entire data set. Mean PC scores were calculated in Microsoft Excel 2003. TpsRelw also allows one to visualize shape differences by creating a deformation grid for each specimen's shape relative to others based on a physical model that minimizes the bending energy required to bend a thin metal sheet (i.e., thin plate spline). The total number of dimensions of variation is $2p - 4$, where p is the total number of landmarks. Since $p = 14$, twenty-four principal component axes were generated. Mean scores along the first principal component axis (PC1) for all samples in this analysis are listed in Table 2.

SPSS version 11.0.0 (SPSS Inc., Chicago, IL, USA, 2001) was used to perform a Discriminant Function Analysis (DFA) on values along PC1 to test for differences among ecotypes for all samples and between sexes within one sea-run and four lake samples.

As a check of the analyses using all samples, a separate PCA and DFA were performed for the subset of ten potentially independent lake samples. In addition, for this subset of ten samples, variation for telencephalon shapes was noticeably different between ecotypes. We tested for these differences in variance between generalists and benthics and among samples within each ecotype using Bartlett's test in Biomstat version 3.30o (Rohlf, 2002). Sample

variance for telencephalon shapes was calculated as the sum of the variances of each of the 24 PC's in a sample.

To test for confounding effects of eye size on telencephalon shape in the subset of ten samples, we used TpsRegr version 1.31 (Rohlf, 2005) to carry out a multivariate regression of the fourteen-landmark array for left telencephalon shapes with natural log-transformed left eye diameter as the independent variable.

Univariate and Bivariate Analyses of Potentially Independent Samples

We performed univariate and bivariate statistical analyses only on the subset of ten potentially independent lake samples. We extended statistical analysis to this subset of ten samples because any samples within it from the same ecotype came from separate drainages and therefore, were unlikely to be pseudoreplicates. All linear morphological measurements and centroid size were natural log-transformed. Biomstat version 3.30o was used to carry out correlation analysis between BL and SL and ANCOVAs of telencephalon centroid size with BL or SL as the covariate. In this subset of ten samples, the assumptions for an ANCOVA were violated. As an alternative method to analyze size-adjusted telencephalon centroid size, we calculated separate regressions for each lake sample. We compared values for predicted telencephalon centroid size at the collective mean for standard length (SL=46.08 mm) or for brain length (BL=4.63 mm) because the rank order of samples did not change much at these or greater length values (see Fig. 3A). Although sea-run fish mature within SL=55-70 mm, almost all derived lake threespine stickleback mature within SL=40-50 mm. Therefore, these values are very good estimates for mature fish in lake populations. Standard error of predicted telencephalon centroid size was calculated as $\sqrt{(s^2_{Y.X} (1/n + ((X_i - \bar{X})^2/\sum x^2)))}$, where $s^2_{Y.X}$ is the

unexplained mean square that summarized the residual variation (i.e., sum of squared deviations divided by df = n-2; Sokal & Rohlf, 2001). Sample differences in predicted telencephalon size values were tested with the GT2-method for unplanned comparisons using Biomstat version 3.30o (Rohlf, 2002), which tests for all differences among samples and is used when sample sizes are unequal. In this method, upper and lower 95% comparison limits are calculated such that each sample's predicted telencephalon centroid size can be declared significantly different from each other at a 5% experimentwise error rate.

RESULTS

Telencephalon Shape Analysis of 19 Populations

The first two principal components accounted for 81.78% of the variance in telencephalon shape, with the first principal component (PC1) accounting for 62.80% (Fig. 2A). Toward positive values of PC 1, the telencephalon outline appears to be triangular, with a concave dorsolateral region of the telencephalon (Dl), and longitudinal elongation, especially between LM 6 to 12. At the negative end of PC1, the outline of Dl is convex, and there is longitudinal compression and strong lateral extension between landmarks 4 to12. Mean telencephalon shapes of generalist samples are distributed along most of PC1, except for the most extreme negative values, which are occupied exclusively by benthic samples.

PC2 accounted for another 18.98% of the shape variation. Positive values of PC2 indicate lateral extension mostly near LM 5 to 9, yielding an overall shape that is more longitudinally compressed and more triangular than lower values. Negative PC2 values represent longitudinal elongation. Although PC2 appears to distinguish ecotypes better than PC1 does, PC1 alone captures more than half of all shape variation. Benthic telencephalon shapes are limited almost

entirely to a single quadrant (negative PC1, negative PC2), from which generalist samples are absent, and mean telencephalon shapes occur in the other three quadrants of the PCA.

The mean telencephalon shape of the sea-run (Rabbit Slough) sample shares with benthics a negative value of PC1 and has a slightly positive but similar value of PC2. Thus, the telencephalon is convex and laterally extended, similar but not identical to that of benthic populations.

We had difficulty classifying the Kashwitna Lake population ecologically and analyzed a sample from a stream population (Little Meadow Creek) to include a broader ecological range. Relative littoral area suggests that the Kashwitna Lake population should be strongly benthic, but trophic morphology and body shape indicate that it is more dependent on plankton. The Little Meadow Creek sample is located far from the benthic samples, but Kashwitna Lake is closer to them in the morphospace, conforming more closely to expectations based on relative littoral area of the lake than to trophic and body-shape traits.

DFA detected a difference between the telencephalon shape of benthics from that of the sea-run (Rabbit Slough) sample (χ^2=33.787, $p<0.05$) and from that of generalists (χ^2=140.523, $p<0.05$).

Analysis of Ten Potentially Independent Populations

Populations from geographically adjacent lakes within a single drainage (Table 2) may share a common freshwater ancestor. Thus, their similarities for brain morphology may be influenced by common ancestry, not similar adaptation, and therefore are not statistically independent observations. Thus, we repeated shape analyses using samples from only five lakes with extreme benthic stickleback (Corcoran, Mud, Walby, Willow, and Tern lakes) and five

lakes containing generalists with the most extreme limnetic traits in our sample of populations (Matanuska, Long, Lynne, South Rolly, and Stormy lakes). No two samples from the same putative ecotype in this subset came from the same drainage. Since samples within each ecotype can be considered potentially independent, this subset was used for additional analysis of relative telencephalon centroid size. We also tested for differences in intrapopulation variation of telencephalon shape between benthics and generalists. Finally, we tested for an association between eye morphology and forebrain shape.

Telencephalon Shape

Shape analysis of the telencephalon was replicated with the subset of ten samples. A PCA of this reduced set of ten samples produced nearly identical results to the PCA with all 19 samples. PC1 and PC2 accounted for 63.83% and 18.83% of the variation, respectively. The positions of these ten potentially independent samples within the plot were virtually unchanged compared to their positions in the full analysis. The variance for telencephalon shapes was greater in generalists than in benthics ($X^2_{c\ 0.5[1]}$=17.423, $p<0.05$), but there were no differences in variance among samples within the benthic ($X^2_{c\ 0.5[4]}$=6.078, p=0.19) or generalist ($X^2_{c\ 0.5[4]}$=4.891, p=0.30) ecotypes.

Telencephalon Size

Differences in relative telencephalon centroid size between benthic and generalist ecotypes were tested using ANCOVA. Samples belonging to the same ecotype were pooled. Compared to benthics, generalist fish had greater relative telencephalon centroid size, using SL as the covariate ($F_{1,278}$=54.377, $p<0.05$), but no differences were found using BL as the covariate

($F_{1,278}$=0.187, p=0.67). However, BL is only moderately correlated with SL within the limited range of sizes measured (r=0.644, p<0.05).

A detailed analysis among samples could determine whether the ecotypic difference for relative telencephalon size using SL as the covariate was due to contributions by all or only a subset of extreme generalist samples. Among the ten potentially independent samples, slopes of regressions for telencephalon centroid size on BL ($F_{9,262}$=3.376, p<0.05) or SL ($F_{9,262}$=4.637, p<0.05) were significantly different. Therefore, we could not use an ANCOVA to test for relative differences in telencephalon size among samples. To size-adjust telencephalon size, we calculated separate regression equations for each lake population using SL or BL as the independent variable and compared their predicted telencephalon centroid sizes (see materials and methods). Unfortunately, regressions using SL for the sea-run (Rabbit Slough) and South Rolly Lake samples were not significant. The covariate used and their values both influenced the rank order of relative telencephalon size across populations, but major differences in rank were not found at or above the collective mean for either covariate (Fig. 3A). When SL is used to adjust size, Matanuska, Mud, Lynne, Tern, and Long lake samples had the highest ranks at one standard deviation below the mean SL (i.e., 41.16 mm). However, rank was different at the collective mean (SL=46.08 mm) but stable up to one standard deviation above (SL=51.58 mm), with the exception of the Matanuska Lake sample, which fell by one rank. When BL is used, South Rolly, Mud, Lynne, and Stormy lake samples had the highest ranks at one standard deviation below the mean (BL=4.17 mm). Rank was different but stable between the mean (BL=4.63 mm) and one standard deviation above the mean (BL=5.14 mm), with the exception of the Matanuska Lake sample, which rose six positions, and Stormy Lake, which fell by seven.

Since the comparison of regression equations in the previous analysis cannot detect statistically significant differences for relative telencephalon centroid sizes among the ten samples, we compared predicted values for telencephalon centroid size based on the collective mean for SL or BL because sample rank was generally stable at these and greater values. Using the GT2-method for multiple comparisons, statistically significant differences were summarized with 95% comparison limits for each sample (Fig. 3B). When SL is used, the most striking result is that Lynne and Matanuska generalist lake samples had the greatest relative telencephalon centroid size. When BL is used, South Rolly and Lynne generalist lake samples had the greatest relative size. Regardless of whether telencephalon size was adjusted using SL or BL as the covariate, lake samples with the largest telencephala were generalists. Compared to benthics, the remaining generalist samples were either intermediate in relative telencephalon size when size-adjusted to SL or small when size-adjusted to BL.

Eye Diameter

The concave shape of the telencephalon in the most extreme generalists based on body shape, diet, and lake bathymetry may be due to obstruction by the orbit or eye socket. A multivariate regression on the ten potentially independent populations using tpsRegr 1.31 (Rohlf, 2005) for the fourteen-landmark telencephalon array on natural log-transformed left eye diameter was significant ($F_{24, 6696}$=1.988, $p<0.05$), but the overall measure of fit was very low (0.70%). Furthermore, there was no correlation between PC1 (i.e., magnitude of telencephalon convexity between about LM 4-12) and natural log-transformed left eye diameter (t_s=0.653, p=0.51). Even though pooling individuals among populations could have produced a spurious association based

on population membership, lack of an association is unambiguous. Therefore, eye size does not appear to influence telencephalon shape variation strongly in adult stickleback.

Sexual Dimorphism

ANCOVA was carried out for the five largest samples to test for sexual dimorphism in relative telencephalon centroid size using SL or BL as covariates. BL was also analyzed using SL as the covariate. When significant size differences were present, males invariably had larger relative telencephalon centroid size and BL (Table 3). There was no detectable telencephalon shape dimorphism in the Long Lake sample, but PCA's of telencephalon shape within samples from the other four sites produced two groups, with the males always displaced slightly, but significantly, toward positive values of PC1, the direction characteristic of generalists' telencephalon shape. However, sex differences in telencephalon shape were not as great as interpopulation differences.

DISCUSSION

The Clever Foraging Hypothesis proposes that neuroanatomical complexity evolves in response to the demands of strategic foraging (Parker & Gibson, 1977; Striedter, 2005). We tested this hypothesis by examining telencephalon morphology in threespine stickleback from ecologically and phenotypically diverse populations from two separate geographic regions of Alaska, the Matanuska-Susitna Valley and the Kenai Peninsula. Lake stickleback may be preyed upon by a variety of fishes, birds, and aquatic insects (Reimchen, 1994). Predatory birds and insects are present in all lakes. We used only populations sympatric with native predatory fishes so that the potential effects of predator avoidance behavior on telencephalon morphology would

be more-or-less constant among populations (Huntingford, 1976; Huntingford *et al.*, 1994; Portavella *et al.*, 1998, 2003, 2004). Unlike cichlids (van Staaden *et al.*, 1994/95; Huber *et al.*, 1997; Pollen *et al.*, 2007) and coral reef fishes (Bauchot *et al.*, 1977), benthic stickleback populations that presumably experience greater habitat complexity have smaller telencephala. However, telencephalon shapes of benthics and the sea-run sample are more convex laterally compared to generalists. Telencephalon morphology is also sexually dimorphic, with larger and less convex telencephala in males. Our study is the one of the first to detect ecotypic divergence of telencephalon morphology among conspecific, field-caught populations of a teleost fish (see Burns & Rodd, 2008), but interpretation of our results is complicated by several factors that we discuss below.

When samples belonging to the same ecotype were pooled, generalists had larger relative telencephalon centroid sizes after adjusting for SL but not BL. However, this analysis using pooled data cannot determine whether this difference was due to contributions by all or only a subset of extreme generalist samples. Tests for differences among samples supported the latter, but no further ecotypic pattern emerged. Although we expected both covariates to generate similar results, SL and BL were moderately correlated. When SL is used as the covariate, three of the top five samples were generalists (i.e., Lynne, Matanuska, Long lakes, Fig. 3). Using body mass rather than SL as a measure of size would have only exaggerated the difference between benthics and the generalist samples with greater relative telencephalon centroid sizes because benthics have greater body size (and presumably greater body mass) per unit SL than more planktivorous stickleback (Baumgartner *et al.*,1988; Walker, 1997; Aguirre, 2007). When mean BL was used as the covariate, two generalist populations had the greatest values for relative telencephalon size, but Long and Matanuska lakes declined several ranks (Fig. 3B). Since the

evolution of body size has become decoupled from absolute brain size in many vertebrate groups (see Striedter, 2005), BL should be the more reliable covariate. However, our data still suggests that extremely divergent ecotypic traits are not good indicators of relative telencephalon size for stickleback, and, therefore, we must reject the CFH at the level of the telencephalon in this species. However, ecotypic differences in telencephalon shape (see below) suggest that subregions within the telencephalon may vary in a size-specific manner.

Population differences in mean relative telencephalon size could be due to differences in cognitive map use, a spatial learning strategy that is processed in the dorsolateral region (Dl, Fig. 1B) in the teleost telencephalon (Salas *et al.*, 1996; Vargas *et al.*, 2000). In cichlids, Dl is larger in species that occupy spatially complex habitats compared to species that live in more open areas (Shumway, 2008). In the threespine stickleback, the Dl region is large and forms the lateral margin of the telencephalon from a dorsal perspective (Nieuwenhuys, 1959; Ekström & van Veen, 1983, 1984; Ekström *et al.*, 1986, 1990; Fig. 1A, B). Dl is also a potential homolog of the tetrapod hippocampus (Rodríguez *et al.*, 2002; Broglio *et al.*, 2003; Northcutt, 2006), which processes spatial information (O'Keefe and Nadel, 1978), but this homology is controversial (Saito & Watanabe, 2006).

In birds and mammals, the size of the hippocampus is associated with residence in structurally complex habitats and food caching (Sherry, 1998). Strictly limnetic British Columbian populations of *G. aculeatus* are less likely to use spatial cues than benthics to forage (Odling-Smee & Braithwaite, 2003a, Odling-Smee *et al.* 2008). Alaskan generalist populations are not as specialized for planktivory as limnetic populations in British Columbia (J. Baker & S. Foster pers. comm., 2008). It is plausible that while generalists with smaller relative telencephalon sizes may depend on plankton for much of their diet using landmarks less often

than benthic specialists, generalists with larger relative telencephalon sizes may be utilizing and integrating multiple learning strategies (see López *et al.*, 1999) because they also use the littoral zones, where benthic prey is available.

Cognitive map use can not account for all relative telencephalon size variation. In pelagic sharks and teleosts, there is considerable variation in relative telencephalon size (Lisney & Collin, 2006), and larger telencephala may be explained by complex social behaviors such as pair formation, communication, or group interaction (Lisney & Collin, 2006; Pollen *et al.*, 2007). Benthic and limnetic stickleback populations differ drastically in their tendency to form shoals (Kozak & Boughman, 2008), courtship rituals (Foster, 1988, 1994a, b, 1995) and in their responses when approached by large groups of conspecifics (Foster, 1988, 1994a, b, 1995). It is not clear how these social behaviors could influence telencephalon evolution in stickleback, but ablation of the stickleback telencephalon in mature male stickleback caused uncoordinated sequencing of courtship and nesting behaviors (Schonherr, 1955; Segaar, 1956) and decreased aggression (Segaar, 1965). Further study on inter-population social differences and their relationships to telencephalon evolution may resolve present ambiguities (see Huber *et al.*, 1997; Pollen *et al.*, 2007; Gonzalez-Voyer *et al*., 2009).

Only a few studies with teleosts have considered intraspecific differences of telencephalon shape (see Burns & Rodd, 2008; Burns *et al.*, 2009). Unlike relative telencephalon size, telencephalon shape is associated with ecotypic foraging mode in threespine stickleback. In PCA plots for our full data set and for the selected subset of ten samples, benthics are restricted to negative values of PC1, which correspond to a convex shape (Fig. 2A). Benthics occur in lakes with high RLA values, which are associated with greater habitat complexity, and with depths of mostly less than 3 m. In contrast, generalist populations are widely distributed along

PC1. Generalist samples from South Rolly, Matanuska, Lynne, and Long lakes have the highest mean values for PC1. Interestingly, these lakes also have very similar morphometry, with steeply sloping basins and maximum depths between 18 m to 24 m. Stickleback from these lakes forage mainly on plankton and have extreme limnetic body shapes, but the South Rolly generalist population is the most specialized for planktivory (J. Baker pers. comm., 2008). Lynne Lake generalist stickleback have intermediate telencephalon shapes, but stomach content data (J. Baker pers. comm., 2008) and foraging behavior (S. Foster pers. comm. 2006; J. Baker pers. comm. 2008) indicate that this population has recently begun to feed on benthos. Putatively generalist populations with telencephalon shapes similar to benthics include Crystal, Beaverhouse, Lynda, and Big lakes. The latter three lake populations occur within the same drainage. Stickleback in Beaverhouse Lake have limnetic body shapes but forage on benthos, and Lynda Lake stickleback have intermediate numbers of gill rakers, intermediate dental microwear, and nearly half of their diet includes benthic prey (Table 1). Clearly, these populations do not have as extreme limnetic qualities compared to the five generalist populations chosen for further analysis. Big Lake stickleback have limnetic body shapes, but this lake has properties that are very different from other deep lakes that we sampled. Since the surface area of Big Lake is ten times greater than that of any other lake sampled, it may have greater variation in microhabitats and predation regimes. In addition, all other lakes sampled in the Fish Creek drainage (Table 2) are upstream and drain into Big Lake, potentially making this stickleback population more susceptible to gene flow than neighboring generalist populations (Aguirre, 2007). Unfortunately, we have very little trophic data for fish from Crystal Lake. Further study may reveal why some generalist lakes contain fish with benthic telencephalon morphology. The Kashwitna Lake population is anomalous because its relative littoral area and dental microwear

of stickleback suggest that this population should be benthic, but it has intermediate body shape and a limnetic diet.

Based on tooth microwear, stomach contents, lateral head and body shapes, and behavioral observations in the field, greater consumption of plankton appears to be associated with higher values of PC1. However, the concave telencephalon shape found in most generalists is not always associated with greater relative telencephalon size. Mud and Corcoran benthic lake stickleback, which have convex telencephalon shapes had relatively large telencephalon sizes (Fig. 3). Therefore, our data suggests that telencephalon shape differences may be better than relative telencephalon size to distinguish ecotypes.

Compared to benthics, generalists have statistically greater variation for telencephalon shapes. Among *Hybopsis* minnow species, variability in brain lobe morphology was greatest in species inhabiting the most variable habitats (Davis & Miller, 1967), and threespine stickleback populations that consume a wide range of prey may consist of diet specialists that specialize on different prey types (Svanbäck & Bolnick, 2007). Thus, greater variation of telencephalon shapes in stickleback from lakes with greater relative limnetic zones may indicate that generalist populations consist of diverse foraging specialists that use different combinations of plankton and benthos in their diets. Further studies that address long-term foraging tendencies (e.g., stable isotope dating, see Moodie *et al.*, 2007) may clarify the relationship between diet specialization and differences in telencephalon morphology.

Little Meadow Creek contains a stream population, and stream and benthic stickleback from the Matanuska-Susitna Valley have similar but not identical body shapes (Aguirre, 2007). Mean telencephalon shape for the Little Meadow Creek sample is located in the shape space between the convex shapes of benthics and the concave shapes of generalists with extreme

limnetic qualities, which is consistent with the body shape results and information on the diet of benthic and stream stickleback (Hart & Gill, 1994). Compared to field-caught benthic fish from ponds, field-caught stream stickleback are poor at using spatial cues while foraging (Girvan & Braithwaite, 1998; Odling-Smee & Braithwaite, 2003b). The shape differences of the telencephala between our stream and benthic samples suggest that convex telencephalon shape may reflect superior spatial learning during foraging.

Unlike our analysis of relative telencephalon size, a clear ecotypic pattern for independent evolution of telencephalon shape is apparent, but its relevance to the CFH is unclear. Telencephala from benthic stickleback populations are generally more convex within both major geographical regions and each drainage sampled. These shape data are independent of size, unless allometry is present. Convex telencephalon shape suggests that benthic populations have greater volume of Dl tissue than generalists with concave telencephala (see Fig. 3). Thus, greater telencephalon convexity may reflect greater Dl volume which is appropriate for foraging, social behavior, parental behavior, or predator evasion in the littoral zone. Benthics may not have as much Dl as stickleback with a larger telencephalon, but they still may devote a larger proportion of the telencephalon to Dl.

Cranial space and sexual dimorphism are confounding variables that could have influenced our results. Limited cranial space during development could influence teleost telencephalon morphology (see Northcutt, 2002; Striedter & Northcutt, 2006). From a lateral perspective, limnetic stickleback have a shallow head while benthic heads are deeper (Walker, 1997; Caldecutt & Adams, 1998; Kristjansson *et al.*, 2002; Wund *et al.*, 2008). The cichlid *Bathybates* retains large eyes, but they have become oval, allowing expansion of the adductor mandibulae muscles into space above the dentary that would be occupied in related species by

the eye (Barel *et al.*, 1989). It is not known whether similar constraints apply to the eye and brain of threespine stickleback. However, in very small fish like stickleback, the eyes are disproportionately large in fry and may exhibit negative growth allometry throughout much of life (Fuiman, 1983). We are currently investigating whether other aspects of neurocranium morphology are associated with telencephalon shape.

Our data also show that telencephalon morphology is sexually dimorphic in stickleback. In Azorean rock-pool blennies (*Parablennius parvicornis*), Dl of their telencephala was larger in females, which have a larger home range (Carneiro *et al.*, 2001). In mammals (Sherry, 1998; Spritzer *et al.*, 2005) and birds (Sherry *et al.*, 1993, Sherry, 1998; Clayton & Krebs, 1994; Healy *et al.*, 2005), the sex with the larger home range usually has a larger hippocampus. In our sea-run and a sample of four lake populations, males had larger relative telencephala regardless of covariate used to adjust for size (Table 3). Relative BL was also larger in males, which suggests that the telencephalon may not be the only part of the brain that is sexually dimorphic for size. The telencephalon shapes of males were slightly but significantly displaced towards positive values of PC1 (i.e., more limnetic shape). Our telencephalon shape data support findings that male stickleback have limnetic traits, compared to females (Caldecutt & Adams, 1998; Kristjansson *et al.*, 2002; but see Bentzen & McPhail, 1984). The inconsistency between sexual dimorphism and ecotypic telencephalon shape variation in stickleback is paradoxical, but the larger telencephala of male stickleback may be associated with the cognitive demands of male parental care (see Gonzalez-Voyer *et al*., 2009) or utilizing spatial information while maintaining a territory (see Sherry, 1998).

Lack of ancestral behavioral and neuromorphological material has limited interpretation in comparative neuroanatomy (Niewenhuys, 1998a, 1998b; Northcutt, 2002; Striedter, 2005).

Since the sea-run stickleback can be used to infer ancestral brain morphology (see Bell, 1995) and behavioral capabilities, this limitation can be minimized in the threespine stickleback. Although the Rabbit Slough population may not represent all sea-run stickleback populations, there is little variation for body shape (Walker & Bell, 2000) and armor (Bell & Foster, 1994; Colosimo *et al.*, 2005) world-wide among sea-run threespine stickleback populations. They feed on plankton at sea (Mackney & Hughes, 1995) and have trophic morphology resembling limnetics (McPhail, 1994). However, based on regressions using BL, all derived lake populations had greater relative telencephalon centroid size than the sea-run (Rabbit Slough) population (Fig. 3). It is plausible that in freshwater populations, as body size decreased, absolute telencephalon size did not decrease as rapidly as overall brain size (Davis & Miller, 1967). If so, then potentially independent lake populations from different drainages must have evolved larger relative telencephalon size repeatedly as an adaptation to freshwater.

The Rabbit Slough sample also had the most negative value for telencephalon shape on PC1, which corresponds to a very convex shape resembling, but statistically different from, that of benthics. Although local adaptation in Rabbit Slough is possible, the most parsimonious explanation for similarity of benthic populations is retention of the ancestral condition. In contrast, the more concave margin of the telencephalon of generalist populations must have evolved independently many times in response to the demands for generalist foraging or lifestyle. Unlike telencephalon shape, head shape in the sea-run Rabbit Slough population is intermediate between one benthic and one limnetic population (Caldecutt & Adams, 1998), and therefore, head shape evolution alone does not seem to explain the convex telencephala of Rabbit Slough fish. Colosimo *et al.* (2005) showed that genetic variation for an armor trait in freshwater stickleback occurs as a rare, recessive allele in sea-run populations, and this is true of other

morphological traits (Miller *et al.*, 2007; Arif *et al.*, 2009; W.E. Aguirre & M.A. Bell, unpublished data). Furthermore, courtship and paternal behaviors in freshwater stickleback are a subset of those of sea-run fish, and diversification of freshwater populations for these behaviors is based on loss of ancestral traits (Shaw *et al*., 2007). Since the resident lake populations in Cook Inlet have evolved within the last 22,000 years (Reger & Pinney, 1996), it is almost certain that the genetic variation for telencephalon morphology and associated learning behaviors in freshwater populations also existed in the sea-run ancestor. We do not expect other sea-run populations to differ drastically from the Rabbit Slough population for telencephalon morphology, but future studies will include sea-run samples from other sites.

In conclusion, unlike in cichlids (Huber *et al.*, 1997) and reef fishes (Bauchot *et al.*, 1977), we do not find support for the Clever Foraging Hypothesis in Alaskan *G. aculeatus* for the telencephalon. However, ecotypic differences for telencephalon shape suggest that regions within the telencephalon (e.g., Dl) may vary in a size-specific manner with ecotype. Telencephalon shapes appear to be convex in benthics while generalists with extreme limnetic qualities have concave telencephalon shapes. Furthermore, male telencephalon shape consistently resembles that of generalist populations. Our findings are not surprising because the teleost telencephalon is complex, and many factors including sociality, parental care, predation, spatial cognition, ontogenetic differences, and others could have contributed to its evolution (see Bshary *et al.*, 2002; Brown *et al.*, 2006; Gonzalez-Voyer *et al.*, 2009). Seasonal or long-term changes in telencephalon morphology or other brain structures may also occur within or differ among sites. Variation in telencephalon morphology that we and others have reported (Bauchot *et al.*, 1977; Huber *et al.*, 1997; Pollen *et al.*, 2007) was based on phenotypes from field-caught specimens, and the contribution of genetic divergence and phenotypic plasticity to these

differences in *G. aculeatus* is unknown. In ninespine stickleback (*Pungitius pungitius*), laboratory-reared marine populations have larger telencephala than pond populations (Gonda *et al.*, 2009), but domesticated animals (Kruska, 1988) and hatchery (Marchetti & Nevitt, 2003; Kihslinger *et al.*, 2006) and lab-reared fish (Burns *et al.*, 2009) have smaller brain volumes than their wild counterparts. Phenotypic plasticity is important because fishes (Francis *et al.*, 1993; Lema, 2006) and other animals subjected to cognitive deprivation experience irreversible deficits in cognition (Patel *et al.*, 1997; Kempermann & Gage, 1999; van Praag *et al.*, 2000; Rampon *et al.*, 2000; Faherty *et al.*, 2003; Olson *et al.*, 2006) that may be related to brain development. Despite the lack of conclusive findings, our study has revealed that many basic questions in telencephalon evolution remain to be answered (Sober, 1997). Although determining the best neurobiological correlate for behavior remains a great challenge for neurobiology (see Rosenzweig, 1996; Vargas *et al.*, 2000), our study provides insights into the environmental correlates of telencephalon size and shape in threespine stickleback. There is great potential in behavioral genetics (Leil *et al.*, 2003; Sforza & Smith, 2003; Pollen & Hoffman, 2008), and threespine stickleback genomics has emerged as a powerful model to understand the evolution of complex traits (see Gibson, 2005; Kingsley & Peichel, 2007; Cresko *et al.*, 2007). Approaches that combine efficient behavioral assays with phylogenetic, genomic, molecular, developmental, and neurobiological evolution studies may clarify the relevant properties of brain complexity, genetics, development, and function as well as its relationship with the complexity of social, trophic, and other environmental variables (Pollen & Hoffman, 2008).

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Table 1. Criteria for ecotype classification of study populations. When available, relative littoral area of lakes (RLA), number of gill rakers, stomach contents, dental microwear, body shape, and foraging behavior were used to classify ecotypes. Sources of information on lake or stickleback properties are described in the materials and methods. Unavailable data are marked with hyphens.

Population	Region	RLA[†]	Gill Rakers[†]	Stomach Contents[§]	Microwear [§]	Body Shape[¶]	Foraging[¥, £]
Beaverhouse	Mat-Su	Limnetic (16%)	Limnetic (21.3)	(63.3% B, 36.7% L) [‡]	–	Limnetic	–
Big	Mat-Su	Limnetic (1%)*	–	–	–	Limnetic	–
Crystal	Mat-Su	Limnetic (52%)*	–	–	–	–	–
Long	Mat-Su	Limnetic (30%)	Limnetic (21.9)	(32.6% B, 67.4% L)	Limnetic	Limnetic	Limnetic
Lynda	Mat-Su	Limnetic (24%)	Uncertain (20.7)	(42.1% B, 57.9% L)	Uncertain	Limnetic	–
Lynne	Mat-Su	Limnetic (51%)*	–	–	–	–	Limnetic
Matanuska	Mat-Su	Limnetic (10%)*	–	–	–	Limnetic	Limnetic
South Rolly	Mat-Su	–	–	–	–	Limnetic	Limnetic
Corcoran	Mat-Su	Benthic (100%)	Uncertain (20.5)	(79.2% B, 20.8% L)	Benthic	Benthic	–
Jim	Mat-Su	Benthic (100%)*	–	–	–	–	–
Mud	Mat-Su	Benthic (100%)	Benthic (17.97)	(93.4% B, 6.6% L)	Benthic	Benthic	–
Willow	Mat-Su	Benthic (100%)*	Benthic (19.9) [‡]	(57.2% B, 42.8% L) [‡]	Benthic	Benthic	–
Walby	Mat-Su	Benthic (78%)*	–	–	–	Benthic	–
Stormy	Kenai	Limnetic (33%)	Uncertain (20.4)	–	–	Limnetic	Limnetic
Engineer	Kenai	Benthic (91%)	Uncertain (20.3)	–	–	–	Benthic
Tern	Kenai	Benthic (100%)*	–	–	–	Benthic	–

Rabbit Slough	Mat-Su	–	Limnetic (22.4) [‡]	(72.8%B, 27.2%L) [‡]	–	–	–
Little Meadow	Mat-Su	–	–	–	–	Stream	–
Kashwitna	Mat-Su	Benthic (100%)	Uncertain (20.9)	(28.5% B, 71.5%L)	Benthic	Intermediate	–

*M.A. Bell (unpublished data) or [†]Walker (1997)
[†]Walker (1997) or [‡]M.P. Travis (unpublished data)
[‡]M.P. Travis (unpublished data) or [§]Purnell *et al.* (2006)
[§]Purnell *et al.* (2006)
[¶]W.E. Aguirre (unpublished data) or [¥]Baker (pers. comm. 2008)
[£]Foster (pers. comm. 2006) and [¥]Baker (pers. comm. 2008).

Table 2. Sources and composition of threespine stickleback samples. Symbols include FM, fixation method (see Materials and Methods); Year, collection year; n, sample size by sex; SL, standard length; SD, standard deviation, and PC1, sample mean telencephalon shape value for the first principle component from our shape analysis with all 19 samples (Fig. 2A).

Population	**Location**	**Drainage**	**FM**	**Year**	**n♂**	**n♀**	**Mean SL ± SD**	**PC1**
	Lat. (N), Long. (W)							
Beaverhouse	61.574, 149.863	Fish Creek	A	2003	23	8	49.439 ± 4.884	-0.0458
Big	61.533, 149.888	Fish Creek	A	2003	12	2	44.709 ± 2.635	-0.0452
Crystal	61.710, 150.100	Susitna River	A	2003	20	0	49.610 ± 3.521	-0.0353
Long	61.578, 149.764	Fish Creek	B	2002	15	23	47.162 ± 6.032	0.1072
Lynda	61.571, 149.836	Fish Creek	B	2002	7	12	48.736 ± 3.224	-0.0121
Lynne	61.712, 150.039	Little Susitna River	A	2005	28	0	42.433 ± 4.546	0.0263
Matanuska	61.332, 149.134	Wasilla Creek	A	2006	25	13	48.269 ± 4.028	0.1029
South Rolly	61.401, 150.073	Susitna River	B	2002	15	2	47.884 ± 3.697	0.1204
Corcoran	61.574, 149.688	Fish Creek	A	2003	2	16	44.096 ± 7.052	-0.0617
Jim	61.330, 148.550	Knik River	B	1992	2	8	45.234 ± 3.310	-0.0106
Mud	61.563, 148.949	Knik River	A	2003	33	25	47.117 ± 4.195	-0.0426
Walby	61.619, 149.211	Wasilla Creek	A	2003	20	10	46.525 ± 5.591	-0.0680

Willow	61.444, 150.033	Susitna River	B	2002	6	12	46.978 ± 3.310	-0.0036
Stormy	60.771, 151.047	Swanson River	B	2002	2	10	50.623 ± 2.886	0.0197
Engineer	60.479, 150.314	Kenai River	B	1996	12	0	56.623 ± 2.110	-0.0287
Tern	60.533, 149.55	Kenai River	A	2004	24	0	42.630 ± 3.776	-0.0273
Rabbit Slough	61.534, 149.268	Rabbit Slough	A	2003	15	14	68.256 ± 3.571	-0.0715
Kashwitna	61.833, 150.076	Susitna River	B	2002	13	0	48.699 ± 2.337	0.0594
Little Meadow	61.563, 149.826	Fish Creek	A	2003	10	0	46.048 ± 1.898	0.0086

Table 3. Sexual dimorphism in telencephalon and overall brain size. Results for ANCOVA of telencephalon centroid size (TEL) using brain length (BL) or standard length (SL) as the covariate; ANCOVA of brain length with SL as the covariate; and discriminant function analysis (DFA) of telencephalon shapes between sexes in one sea-run (A), two benthic (B), and two generalist (G) populations. All measured values were natural log (LN)-transformed. DV, dependent variable; CVT, covariate.

Site	**DV**	**CVT**	**ANCOVA**	**Adj. Means (LN)**		**DFA**
				male	**female**	
Rabbit Slough (A)	TEL	BL	$F_{1,26}$=36.219, p<0.05	1.355	1.287	χ^2=6.495, p<0.05
	TEL	SL	$F_{1,26}$=21.569, p<0.05	1.374	1.266	
	BL	SL	$F_{1,26}$=13.901, p<0.05	1.825	1.774	
Mud Lake (B)	TEL	BL	$F_{1,55}$=82.493, p<0.05	1.210	1.132	χ^2=35.075, p<0.05
	TEL	SL	$F_{1,55}$=82.780, p<0.05	1.232	1.103	
	BL	SL	$F_{1,55}$=19.498, p<0.05	1.549	1.497	
Walby Lake (B)	TEL	BL	$F_{1,27}$=1.800, p=0.19	—	—	χ^2=6.420, p<0.05
	TEL	SL	$F_{1,27}$=10.930, p<0.05	1.042	0.974	
	BL	SL	$F_{1,27}$=11.177, p<0.05	1.461	1.418	
Long Lake (G)	TEL	BL	$F_{1,35}$=10.960, p<0.05	1.174	1.131	χ^2=2.984, p=0.23
	TEL	SL	$F_{1,35}$=20.460, p<0.05	1.207	1.109	
	BL	SL	$F_{1,35}$=8.731, p<0.05	1.595	1.540	
Matanuska Lake (G)	TEL	BL	$F_{1,35}$=0.766, p=0.39	—	—	χ^2=8.144, p<0.05
	TEL	SL	$F_{1,35}$=26.660, p<0.05	1.305	1.201	
	BL	SL	$F_{1,35}$=27.464, p<0.05	1.671	1.602	

Figure 1. The threespine stickleback brain. (**A**) Dorsal view (anterior is up). From anterior to posterior, olfactory nerves, olfactory bulbs (olf), forebrain (fb), pineal gland (pg), optic lobes (opt), cerebellum (cb), and hindbrain (hb). Ventral structures (pituitary gland and hypothalamus) are not shown. (**B**) Forty μm Nissl-stained cross-section of telencephalon at level of a section (S) in A (dorsal is up). The three major teleost pallial subregions are the dorsomedial (Dm), dorsodorsal (Dd), and dorsolateral (Dl). Endorhinal fissure (ef) and ventral edge of sulcus limitans telencephali (slt) are shown. (**C**) Dorsal view (anterior is left) of the telencephalon showing fixed (1, 14) and semi-landmarks (circles) used in this study.

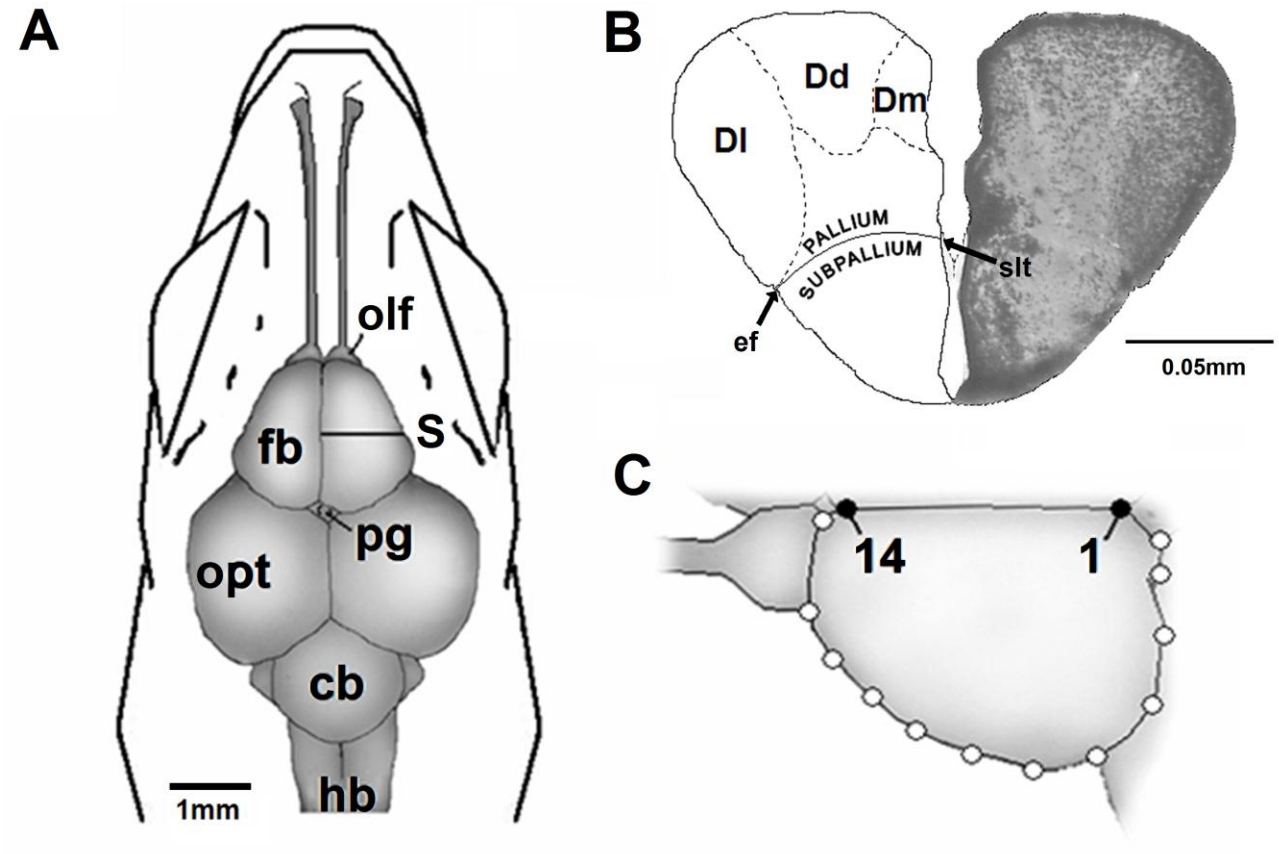


Figure 2. Variation of telencephalon morphology. (**A**) Principal components analysis plot depicting the first two major axes of telencephalon shape variation. Points are sample means from each population. The telencephalon with the area analyzed in this study (shaded) is at the upper right (anterior is left). Deformation grids around the plot in (A) describe shapes at the adjacent ends of each PC axis. The symbols for ecotypes are in the inset legend. The names of populations used for further analysis appear within the plot. (**B**) Lateral views of freshwater stickleback ecotypes and dorsal views of their brains (anterior is left). (i) Benthic stickleback (Walby Lake) showing the deep body and convex telencephalon shape (inset, arrow). (ii) Generalist stickleback (South Rolly Lake) showing the elongate body and concave telencephalon shape (inset, arrow). Scale bars are 10 mm for fish and 1 mm for the brain.

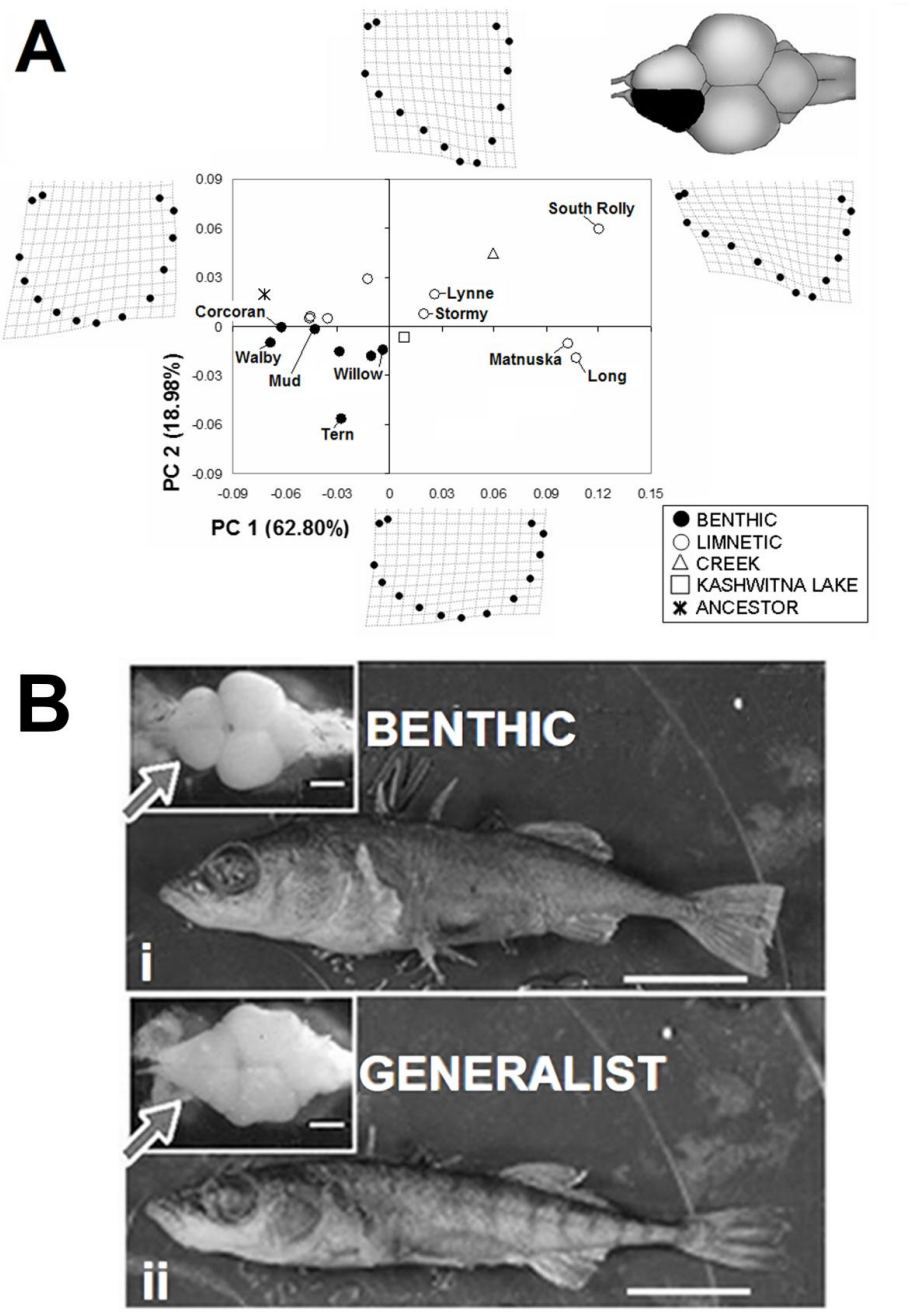
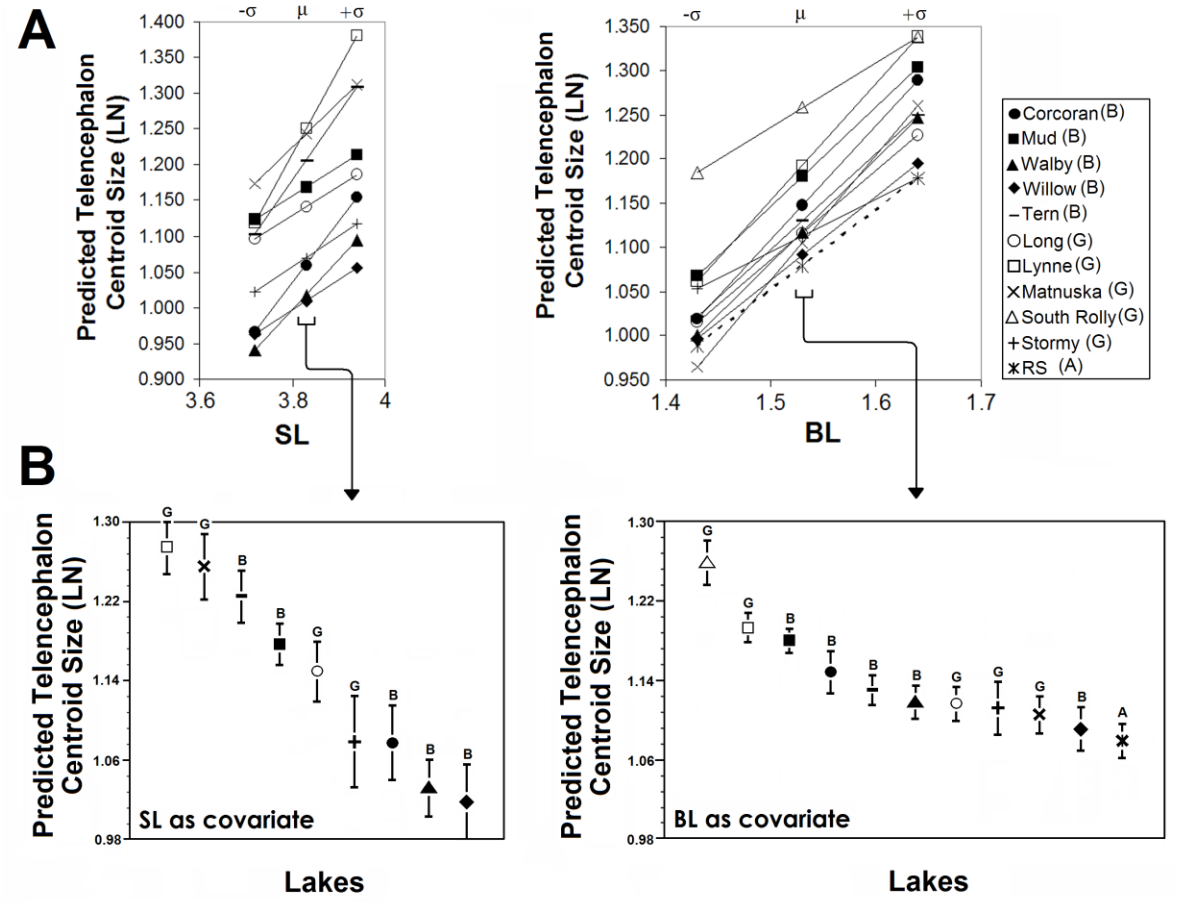


Figure 3. Rank order of relative telencephalon centroid size from ten potentially independent lake populations. (A) Predicted values of telencephalon centroid size based on regressions on standard length (SL) or brain length (BL) for the collective mean (μ) ±1 one standard deviation (σ). Regressions using SL for South Rolly Lake and Rabbit Slough were not significant. Regression line using BL for Rabbit Slough is shown in dotted line. (B) 95% comparison limits from the GT2-method for unplanned comparisons of the predicted telencephalon centroid size using the collective mean of SL=46.08 mm or BL =4.63 mm. All data were natural log-transformed. Solid symbols and ─, benthics; ✕, sea run; all other symbols, generalists. See inset legend in (A) for names of names of sites.



Chapter 4

Phenotypic Plasticity of the Threespine Stickleback (*Gasterosteus aculeatus*) Telencephalon in Response to Experience in Captivity

ABSTRACT

Phenotypic plasticity allows an organism to change its phenotype in response to an environmental change. The present study used threespine stickleback to examine phenotypic plasticity of the telencephalon in relation to inferred ecology. Fish from derived, freshwater populations were sampled from three shallow, structurally complex lakes with benthic-foraging stickleback (benthics) and three deep, structurally simple lakes with planktivores (limnetics). The telencephalon of specimens preserved immediately after capture (field-preserved), field-caught fish held in aquaria for 90 days (lab-held), and lab-bred fish from crosses and raised in aquaria were compared. Field-preserved sea-run (ancestral) stickleback were collected from two separate sites, and parents of lab-bred sea-run stickleback were collected from one of these sites. As in a previous study, an ecotypic pattern is present for telencephalon shape in field-preserved and lab-held fish, with limnetics possessing triangular dorsal shapes and benthics and sea-run fish having rounder shapes, but no such pattern was detected in lab-bred fish. Benthics had larger relative telencephalon sizes than limnetics within each treatment type, and field-preserved sea-run fish had smaller relative telencephalon sizes than field-preserved lake fish. Intra-population analyses of lake samples showed that field-preserved fish consistently had larger relative telencephalon sizes than lab-held and lab-bred fish. In contrast, lab-bred fish from the Rabbit Slough sea-run population had larger greater relative telencephalon sizes than field-preserved counterparts. In a separate study using Corcoran (benthic) and Long (limnetic) lake populations, samples of lab-

held fish were collected at 30, 60, and 90 days to investigate the influence of time in captivity on telencephalon morphology. In both populations, the telencephalon shapes of lab-held fish became less like those of field-preserved fish and more like lab-bred ones over the course of 90 days. In contrast, relative telencephalon size decreased dramatically by 30 days after which there was minimal change. Overall, our results are consistent with the interpretation that the telencephalon of threespine stickleback is phenotypically plastic and that this plasticity occurs in ancestral sea-run populations which was most likely retained in derived resident freshwater populations.

INTRODUCTION

Phenotypic plasticity is the ability of an organism to respond to an environmental alteration with a change in form, state, movement, or rate of activity (West-Eberhard 2003). While the proximate causes of plastic changes to the brain are being actively studied (see Kolb *et al.* 2003; Curley *et al.* 2011; Mongiat & Schinder 2011; Toni & Sultan 2011), the evolution of brain plasticity remains poorly understood (see Nieuwenhuys 1998a,b; Northcutt 2002; Striedter 2005). The telencephalon of threespine stickleback fish (*Gasterosteus aculeatus*) represents a promising model to study the evolution of brain plasticity.

As in other vertebrates, the telencephalon of teleost fishes is the dorsal portion of the forebrain (Fig. 1a, b), and it mediates a variety of behaviors such as classical conditioning (Flood *et al*. 1976; Overmier & Hollis 1983), the processing of sensory information (Davis & Kassel 1983; Hofmann 2001), mating and reproduction (Schonherr 1955; Segaar 1956; Demski & Beaver 2001), social behavior (Huber *et al*. 1997; Kotrschal *et al*. 1998; Hofmann 2001; Pollen *et al*. 2007), aggression (Segaar 1965; Davis & Kassel 1983), schooling behavior (Davis &

Kassel 1983; Shinozuka & Watanabe 2004), and avoidance learning (Portavella *et al.* 2003, 2004; Vargas *et al.* 2009). In the wild, the size of the telencephalon is associated with residence in structurally complex habitats (Bauchot *et al*. 1977, van Staaden e*t al*. 1994/95; Huber *et al*. 1997), superior spatial learning (Vargas *et al*. 2000, 2009), greater parental care (Gonzalez-Voyer *et al.* 2009), monogamy (Pollen *et al*. 2007), and sociality (Pollen *et al*. 2007). Plastic responses of fish telencephalon morphology are also well-documented (e.g., Vargas *et al*. 2000; Marchetti & Nevitt 2003; Kihslinger *et al*. 2006; Burns *et al*. 2009), and norms of reaction vary across fish groups. Hatchery-bred rainbow trout (Marchetti & Nevitt 2003; Kihslinger *et al*. 2006) and lab-bred guppies (Burns *et al.* 2009) have smaller telencephalon sizes than their wild counterparts. In contrast, lab-bred and field-preserved cichlids do not differ in telencephalon size (see Pollen *et al.* 2007).

The threespine stickleback is a suitable model system to study the relationship between phenotypic plasticity of the telencephalon (hereafter referred to as "telencephalon plasticity") and evolution. It is primitively marine or anadromous, and countless independently derived freshwater populations have evolved from anadromous populations (Bell 1976, 1995). Derived lake stickleback populations have adapted to different foraging demands, resulting in ecotypic divergence among populations. Bottom-feeding (benthic) and planktivorous (limnetic) stickleback populations represent extremes along a dietary continuum. Benthics inhabit structurally complex habitats of the littoral zone, while limnetics occur in open-water areas of lakes (Schluter & McPhail 1992). Extreme benthic and limnetic specialists are highly divergent for foraging behavior and morphology. Compared to benthics, limnetics have longer, narrower snouts, more and longer gill rakers (McPhail 1984, 1992,1994), more teeth (Caldecutt *et a*l. 2001), greater armor (Vamosi 2002), a more streamlined body (Lavin & McPhail 1985, 1986;

Walker 1997; Aguirre 2007, 2009) and head (Willacker *et al*. 2010) form, and a more triangular dorsolateral region of the telencephalon (Park & Bell 2010). Limnetics can also forage more effectively on plankton than benthics (Bentzen & McPhail 1984) but have shorter memory for handling prey items (Mackney & Hughes 1995). In addition, compared to benthics, limnetics exhibit more conspicuous courtship behavior (Foster 1994), less male-male aggression (Scotti & Foster 2007), and do not engage in cannibalism of young defended by conspecific males (Foster 1994).

Although morphological traits that vary with diet in stickleback can be influenced by phenotypic plasticity (Meyer 1987; Wimberger 1991; Wund *et al.* 2008), genetically-based ecotypic differences of trophic structures (McPhail 1984, 1992; Lavin & McPhail 1987) and body form (Hendry *et al.* 2002; Spoljaric & Reimchen 2007; Aguirre & Caldecutt, unpublished data) are well-documented. However, to the best of our knowledge, telencephalon plasticity in fishes as it relates to ecotypic variation has never been studied. Experience is important to stickleback learning abilities that are mediated by the telencephalon. For example, spatial learning differences between field-caught pond and river stickleback specimens (Girvan and Braithwaite 1998, 2000; Odling-Smee & Braithwaite 2003a, b) were absent using lab-bred fish (Girvan & Braithwaite 2000). Therefore, telencephalon plasticity may be substantial in this species.

The present study used threespine stickleback from Cook Inlet, Alaska to investigate the derived and ancestral conditions of telencephalon plasticity. To distinguish the importance of genetic and environmental factors on telencephalon morphology, three treatment types were compared. *Field-preserved fish* were sacrificed and fixed in the field immediately upon capture. *Lab-held fish* were caught in the field and then held in aquaria for up to 90 days. Finally, *lab-*

bred fish were produced from artificial crosses and raised in aquaria. Three benthic and three limnetic lake populations were sampled to explore potential ecotypic differences in telencephalon plasticity. These lake populations occur in a recently deglaciated region of southcentral Alaska (Bell *et al*. 1993) that was colonized by anadromous ancestors within the last 15,000 years (Reger & Penny 1996; Bell *et al*. 1993). Compared to the benthics, the limnetic populations inhabit deeper, structurally simpler lakes and have streamlined body (Aguirre 2007) and head shapes (Willacker *et al*. 2010), greater gill-raker number (Walker 1997; Park & Bell 2010, Park unpublished data), planktivore dental microwear patterns (Purnell *et al*. 2006), and planktivorous foraging behavior (S.A. Foster & J. Baker personal communication). Therefore, these benthic and limnetic populations occur toward the opposite ends of a benthic-limnetic continuum for lake populations. The telencephala of these benthics were expected to be laterally convex and similar in relative size to those of limnetics (Park and Bell 2010).

In birds and mammals, individuals raised or held in captivity typically have less neural tissue compared to their wild counterparts in telencephalic nuclei (Buchanan *et al.* 2004; Mirescu *et al.* 2004; Day *et al.* 2008). Similarly, captive-bred fish usually have smaller telencephalon sizes than their field-preserved counterparts (Marchetti & Nevitt 2003; Kihslinger *et al*. 2006; Burns *et al*. 2009), and neural atrophy occurs in captive fishes (Burgess & Coss 1982, Miranda *et al*. 2003, Mirescu *et al*. 2004, Buchanan *et al*. 2004). Thus, for each lake population, lab-held and lab-bred stickleback were expected to have smaller relative telencephalon sizes than field-preserved ones.

In stickleback, contemporary anadromous populations can be used to infer the ancestral condition of traits (Bell 1976, 1995). Field-preserved anadromous fish were collected from two geographically distant sites in Cook Inlet, AK. Lab-bred fish were collected from one of these

sites. Lab-held anadromous fish could not be acquired because of their high mortality in captivity. The sampled anadromous populations should be similar to other anadromous populations because there is little morphological variation among anadromous threespine stickleback populations worldwide (Bell & Foster 1994; Walker & Bell 2000; Colosimo *et al*. 2005), and the fossil record indicates that morphological traits of marine threespine stickleback have not changed considerably for approximately 13 million years (Bell 1994; Bell *et al*. 2009). The patterns of phenotypic plasticity expressed in ancestral, marine stickleback tend to mirror the patterns present in benthic and limnetic populations for trophic morphology (Wund *et al*. 2008) and courtship behaviors (Shaw *et al.* 2007). Therefore, as in lake populations, telencephalon plasticity should be substantial in anadromous populations, and lab-bred anadromous stickleback were expected to have smaller relative telencephalon sizes than their field-preserved counterparts.

MATERIALS AND METHODS

Sampling

Threespine stickleback were collected using 3.18 mm or 6.36 mm mesh unbaited Gee minnow traps set overnight in $\leq$ 1 m depth and $\leq$ 3 m from shore. Water temperatures were between 15° and 20° C. Stickleback samples were collected from three putatively benthic, three putatively limnetic, and two anadromous populations from the Matanuska-Susitna Borough of Cook Inlet, Alaska (Table 1). Ecologically similar populations came from different drainages, and so, similarities among freshwater populations that are divergent from the anadromous ancestor most likely evolved independently (Bell 1995; Taylor & McPhail 1999, 2000; Aguirre 2007). Benthic populations were sampled from Corcoran (61.574°N, 149.688°W), Mud

(61.563°N, 148.949°W), and Walby lakes (61.619°N, 149.211°W). Limnetics came from Long (61.578°N, 149.764°W), Lynne (61.712°N, 150.039°W), and South Rolly (61.401°N, 150.073°W) lakes. Anadromous stickleback were collected from Rabbit Slough (61.534°N, 149.268°W) and Mud Lake (61.563°N, 148.949°W). The resident freshwater population of threespine stickleback in Mud Lake is sympatric with an anadromous population, but the two are reproductively isolated (Karve *et al.* 2008; Bell *et al.* 2010). Park and Bell (2010) presented dietary, phenotypic, geographic, and ecological information for these populations except for anadromous Mud Lake fish, but these fish are phenotypically indistinguishable from Rabbit Slough stickleback (Aguirre *et al.* 2008). Sampling, husbandry, and care of all experimental subjects were approved by the Alaska Department of Fish and Game and by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University.

Up to three treatment types per site were used (Table 1). Field-preserved fish were collected and immediately sacrificed in the field by overdose in MS-222. Samples from lakes were preserved using 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), which is isotonic with fish tissue and buffered at pH 7.4. Anadromous stickleback from Mud Lake and Rabbit Slough were preserved using 3.7 % formaldehyde (=10% formalin) in 0.1 M PBS (see Table 1). Ten percent formalin or 4% paraformaldehyde in 0.1 M PBS are standard fixatives for gross brain morphology and results using either fixative are statistically indistinguishable (see Lisney & Collin 2006, Yopak *et al*. 2007). We validated these results for stickleback using field-preserved samples of anadromous Rabbit Slough fish that were preserved in both fixatives. Anadromous males from Rabbit Slough that were preserved in 3.7 % formaldehyde (N=15, collected in 2003) were compared to a sample of males from the same site preserved using 4% paraformaldehyde (N=15, collected in 2007). Using the results from the common Principal

Components Analysis (see RESULTS below), no differences were detected between fishes preserved in formaldehyde or paraformaldehyde for telencephalon shapes along PC1 (Discriminant Function Analysis: χ^2=0.414, p=0.520, df=1). Similarly, relative telencephalon size did not differ between samples (ANCOVA: $F_{1,27}$=0.441, p=0.512). Thus, both samples of field-preserved Rabbit Slough males could be pooled in subsequent analyses, and samples preserved in 3.7 % formaldehyde could be compared with those preserved in 4% paraformaldehyde (see Table 1).

Lab-held fish were collected from lake populations. These samples included pre-reproductive one-year old fish because of high mortality of senescing, field-caught, two-year old adults during captivity. Live fish were transported to the University of Alaska Anchorage, kept in outdoor pools in aged tap water for 24-48 hours, and prepared for shipping. They were placed in plastic bottles, aerated with an air stone, and cooled down to 5-8° C. The volume of water in the bottles was reduced to about one-third of the total volume, and the bottles were sealed and placed into an ice chest with freezer packs, and shipped overnight to Stony Brook University. Upon arrival, the fish were thermally acclimated over several hours and then transferred to 60 l aquaria maintained at 18°C and 3 ppt artificial saltwater, which was prepared using Instant Ocean® sea salt. The aquaria were filtered continuously with a sponge filter (i.e., Hydro-Sponge II, Aquarium Technology, Inc.) and hanging power filter (i.e., Aquaclear 30 power filter, Rolf C Hagen Corp.). The fish were fed daily with thawed, frozen adult brine shrimp. All lab-held fish were treated prophylactically with an antibiotic (i.e., Nitrofurazone, Aquatrol Inc. Pharmaceutical Division) and a parasiticide (i.e., PraziPro™, Aquascience Research Group Inc.) after two weeks of captivity. Lab-held fish were sampled after 90 days in captivity for all lake populations. However, subsamples from the lab-held fish from Corcoran (benthic) and Long

(limnetic) lakes were also collected at 30 and 60 days. All fish were sacrificed using overdose in MS-222 and preserved in 4% paraformaldehyde.

Lab-bred lacustrine fish came from the six lakes, and lab-bred anadromous fish came from Rabbit Slough. Artificial crosses were performed at the University of Alaska Anchorage. Sexually mature fish collected from the field were used as parents to produce the lab-bred fish. Gravid females were recognized by their swollen abdomen and males by their nuptial coloration. For each population, ten sexually mature fish of each sex were anesthetized in MS-222 and used in a mass cross. Sperm were obtained by cutting open the abdomen of ten males and placing the excised testes into a few drops of 3 ppt artificial seawater in a plastic culture dish. The testes of all ten males were minced and mixed with fine forceps to produce a heterogeneous mixture of sperm. Eggs were obtained from ten females by gently squeezing the abdomen and expelling the eggs into a few drops of 3 ppt artificial saltwater in a plastic culture dish. Sperm were pipetted from the minced testis preparation over the eggs. The eggs were examined periodically, and a fertilization membrane could be observed within a few minutes in successfully fertilized eggs. Eggs were washed repeatedly by pipetting 3 ppt artificial saltwater over them and removing the fluid, testis debris, and ovarian mucous. They were kept in plastic culture dishes with 3 ppt artificial saltwater in a constant-temperature incubator at 19º C. Within three days after fertilization, the eggs were transferred to 35 or 50 ml vials nearly filled with 3 ppt artificial saltwater at 19^{o} C, chilled to 5º C, and shipped in a styrofoam box with freezer packs to Stony Brook University. Assuming that the sperm from all testes had equal potency and that embryo mortality was random, the embryos produced should include a mixture of individuals with diverse genotypes that are representative of the source population. Approximately forty random embryos were transferred into each of five plastic culture dishes with 3 ppt seawater and placed

into an incubator at 19º C. Water was changed daily. Three to five days after the fry hatched and their yolk sacs were absorbed, they were fed live brine shrimp nauplii daily. Fourteen days after hatching, the fry were transferred from culture dishes to bare 60 l aquaria. Once all lab-bred fish reached 10 mm standard length (i.e., distance from tip of snout to end of vertebral column), they were switched to a daily diet of thawed, frozen adult brine shrimp. All lab-bred specimens were sampled at fourteen months post-hatching. All fish were sacrificed using overdose in MS-222 and preserved in 4% paraformaldehyde.

Brain Imaging and Geometric Morphometrics

The methods used to digitize and place landmarks on the dorsal aspect of the telencephalon are explained in detail in Park and Bell (2010). Briefly, the brains of specimens were extracted dorsally using fine forceps. Each brain was stored in a 1.5 ml tube with the same fixative used to preserve the fish. The remaining body of each specimen was rinsed briefly in deionized water and stored individually in a 20 ml scintillation vial with 50% isopropyl alcohol in deionized water.

Digital images of the dorsal aspect of the brain were taken using a computer-assisted video image analysis system, Sony DXC-390 video camera, mounted on a Leica MZ75 microscope. Images were acquired using ImagePro software (version 4.1.0.0 for Windows 95/NT/98). Magnification on the microscope was always set to 6.3X, and images were saved in TIF format.

Geometric morphometric methods (Rohlf & Marcus 1993; Zelditch et al. 2004) were used to study telencephalon shape variation of only the left lobe (Fig. 1). Using tpsDig version 2.05 (Rohlf 2006), a pair of extreme landmarks (i.e., LM 1, LM 14) was fixed to defined points,

and 12 intervening semi-landmarks (Bookstein 1991; Bookstein 1996/97; see below) were digitized along the lateral edge of the left telencephalon lobe at approximately even intervals. The fourteen landmarks were placed successively at the following points (Fig. 1c): LM 1, posteromedian juncture of the right and left telencephalon lobes (fixed); LM 2, most posteromedian edge of telencephalon where it abuts the left optic lobe; LM 3, median edge of sulcus ypsiloniformis; LM 4, lateral edge of sulcus ypsiloniformis; LM 5, midpoint between LM 4 and 6; LM 6, most posterolateral edge of telencephalon where it abuts the optic lobe; LM 7 to LM 11 were at equal intervals between LM6 and LM 12; LM 12, most anterolateral edge of telencephalon where it abuts olfactory lobe; LM 13, anteromedian edge of telencephalon where it abuts olfactory lobe; LM 14, anteromedian juncture of the right and left telencephalon lobes where they abut the olfactory bulbs (fixed).

The semi-landmark data were "slid" and aligned using Procrustes superimposition, as implemented in tpsRelw version 1.42 (Rohlf 2005b), to eliminate variation from rotation, translation, and size. The semi-landmark technique allows superimposition of shapes where there are no fixed landmark points that can be accurately digitized, but it requires that the terminal landmarks (LM 1 and LM 14) remain fixed (Bookstein 1996/97). Each intermediate semilandmark can "slide" parallel to a line connecting the semi-sliding landmark to the points on its immediate left and right. TpsRegr 1.31 (Rohlf 2005a) was used to get telencephalon centroid sizes from the landmark data. Centroid size is the square-root of the sum of squared distances of each landmark from the midpoint of all 14 landmarks. Unlike linear size measures, it takes distances in multiple directions into account. The online supplement for Park and Bell (2010) provides validation studies indicating that centroid size using these landmarks is a reliable proxy

for telencephalon volume, that size and shape of the left and right telencephalon lobes are not statistically different, and that time in the fixative does not affect telencephalon morphology.

Multivariate Analysis of Telencephalon Shape

TpsRelw version 1.42 (Rohlf 2005b) was used to perform a Principal Components Analysis (PCA) of the landmark data. PCA summarizes shape variation in morphospace by a low-dimensional Euclidean space representing as much of the variation as possible. All specimens (Table 2) were included in a single alignment from which the shape variables (i.e., partial warps and uniform component) were generated. With $p = 14$ landmarks, there were $2p - 4 = 24$ shape variables. TpsRelw also allows visualization of shape differences by creating a deformation grid for each specimen's shape relative to others based on a physical model that minimizes the bending energy required to bend a thin metal sheet (i.e., thin plate spline). The deformation grid represents the smoothest deformation that can describe the observed shape differences. It was composed of deviations from the grand mean shape. Mean PC scores were calculated in Microsoft Excel 2003; mean scores along the first principal component axis (PC1) for all samples are listed in Table 1. SPSS version 11.0.0 (2001) was used to perform a Discriminant Function Analysis (DFA) on values along PC1 (or PC2) to test for differences between pairs of samples.

Selection of the Standardizing Variable

The telencephalon is typically size-standardized using a measure of body size or brain size (see Striedter 2005). Statistical analyses were used to determine the better of the two covariates for our data (Fig. 2). Body size was estimated using the centroid size based on a

standard configuration of landmarks located around the lateral edge of the body. Overall brain size was estimated using the dorsal area of the brain.

To calculate centroid body size, the left lateral side of each specimen was photographed using a 3.3 megapixel Olympus Camedia C-3000 digital camera. Data were collected and analyzed using the same TPS-series software programs already mentioned. Body size was estimated from the centroid size using 14 out of the 15 outer landmarks employed by Walker (1997); landmark 2 (i.e., supraoccipital notch immediately lateral to the dorsal midline) could not be used in the present analysis because the supraoccipital notch was always damaged during brain extraction. Landmarks were digitized using tpsDig version 2.05 (Rohlf 2006), and centroid sizes for the corresponding body shapes of all specimens were acquired using TpsRegr version 1.31 (Rohlf 2005a) and natural-log transformed. A separate, preliminary analysis using whole specimens did not detect statistical differences between natural-log transformed body centroid sizes estimated with or without landmark 2, and another study indicated that the pattern of covariation between natural-log transformed body centroid sizes and body weights did not differ among multiple benthic and limnetic lake populations (Park unpublished data; also see Berner 2011). Thus, natural-log transformed body centroid size calculated using the 14 landmarks was considered a suitable measure of stickleback body size.

Park and Bell (2010) used the length of the brain between the anterior end of the telencephalon and the posterior end of the cerebellum to standardize telencephalon size, but this measurement does not take into account potential variation across the lateral width of the brain. Thus, the dorsal area of the brain that is intersected by this length, which includes the telencephalon lobes, optic lobes, and the cerebellum, was measured using SigmaScan Pro Image Analysis Software version 4.01.003 (SPSS Inc. 1987-1997). The outer edges of these structures

were traced and their inclusive area was calculated for each brain. The olfactory bulbs or hindbrain were not included in this measurement because the maximum number of specimens was desired, and these structures were often damaged during extraction. The posterior boundary of the hindbrain was also difficult to identify from the dorsal view, which made it an unreliable posterior boundary for measurement. Our measure of dorsal brain area was considered a suitable proxy for overall brain volume because a preliminary analysis using a subset of data from all six lake populations indicated that this measure is a reliable correlate of whole brain area calculated from lateral aspect, and the pattern of covariation between the two variables did not differ among lake populations (see Appendix 3).

Biomstat version 3.30o was used to carry out correlation analysis between dorsal brain area and centroid lateral body size for each lake and anadromous sample of field-preserved fish. All measurements were natural log-transformed. These results are summarized in Table 2. The association between body and brain size covariates was statistically significant for lake samples and correlation coefficients ranged from 0.742 to 0.962. However, the association between brain area and body centroid size was not statistically significant for anadromous samples, which indicated that the use of one size covariate would generate a different result compared to using the other (see Park & Bell 2010). Brain area should be the more reliable covariate than body centroid size because the three brain structures used to estimate it are anatomically and functionally integrated to a greater extent than body size is to the telencephalon (see Broglio *et al*. 2003; Rodríguez *et al*. 2005), and in many vertebrate groups, the body size has become decoupled from absolute brain size (see Striedter, 2005). Thus, brain area was used to size-standardize the telencephalon in all subsequent analyses.

Relative Telencephalon Size - Univariate and Bivariate Statistical Analyses

Univariate and bivariate statistical analyses were performed on neuroanatomical traits. All measurements were natural log-transformed, and the telencephalon was size-standardized using dorsal brain area. Statistical analyses were conducted using Biomstat version 3.30o. ANCOVA was used to test for ecotypic differences of relative telencephalon size within field-preserved, lab-held, or lab-bred treatments. Similarly, ANCOVA was used to detect population differences within treatments, which, if present, were distinguished using the GT2-method test for unplanned comparisons (Rohlf 2002). In this method, upper and lower 95% comparison limits are calculated such that each sample's mean relative telencephalon centroid size can be declared significantly different from all others at a 5% experiment-wise error rate.

The same statistical methods were employed to test for intra-population differences. ANCOVA was used to detect differences in relative telencephalon sizes among field-preserved, lab-held, and lab-bred samples within a lake population. Differences among samples were distinguished using the GT2-method test for unplanned comparisons. In contrast, the slopes of regressions for field-preserved and lab-bred Rabbit Slough anadromous samples were statistically different, which violated the assumptions of an ANCOVA. Therefore, size-adjusted telencephalon sizes among anadromous samples were compared using methods described in Park and Bell (2010). Briefly, for each population, a sample regression equation was calculated and used to generate a predicted mean telencephalon centroid size value from the collective brain area mean of both anadromous samples (17.841 mm^2). A t-test was used to compare predicted means.

RESULTS

Telencephalon Shape - PCA Using All Samples

A Principal Components Analysis (PCA) of telencephalon shapes was performed using all samples (Table 1). The first principal component (PC1) accounted for 45.68% of the variation and the second (PC2) accounted for 26.91% (Fig. 3). Towards positive values of PC1, the telencephalon outline is concave, triangular, and elongate along the dorsal midline. At the negative end of PC1, the outline of the telencephalon is convex and round, and there is longitudinal compression and lateral extension between landmarks 4 to 12. Positive values of PC2 also indicate longitudinal compression and lateral extension mostly near landmarks 5–9, yielding an overall triangular shape that is less elongate than shapes at the positive end of PC1. Negative PC2 values represent elliptical shapes with longitudinal elongation. While both PC1 and PC2 appeared to distinguish field-preserved from lab-held and lab-bred samples, only PC1 distinguished ecotypes (see below).

Telencephalon Shape - Field-Preserved, Lab-Held (90-day), and Lab-Bred Samples

Telencephalon shapes of field-preserved fish exclusively occupied a single quadrant (positive PC1, positive PC2; Fig. 3). Both 90-day lab-held and lab-bred samples occurred along more negative values of both PC axes than field-preserved ones. Along PC1, Discriminant Function Analysis (DFA) distinguished the telencephalon shapes of field-preserved fish from those of lab-held (χ^2=130.679, p<0.001, df=1) and lab-bred fish (χ^2=68.747, p<0.001, df=1), and telencephalon shapes between lab-held and lab-bred fish were also different (χ^2=11.212, p<0.001, df=1). Similarly, along PC 2, telencephalon shapes differed between field-preserved

and lab-held fish (χ^2=55.447, p<0.001, df=1), field-preserved and lab-bred fish (χ^2=99.207, p<0.001, df=1), and lab-held fish and lab-bred fish (χ^2=13.934, p<0.001, df=1).

A DFA detected a difference in telencephalon shapes between benthics and limnetics along PC1 for field-preserved (χ^2=13.253, p<0.001, df=1) and lab-held (χ^2=3.883, p<0.05, df=1) samples. The general pattern within both treatment groups was similar, with benthics having rounder shapes than limnetics for both treatments. However, no ecotypic pattern was detected for lab-bred samples (χ^2=1.057, p=0.304, df=1). Because PC2 did not distinguish ecotypes in field-preserved (χ^2=0.822, p=0.365, df=1), lab-held (χ^2=0.0249, p=0.875, df=1), or lab-bred (χ^2=3.02, p=0.0824, df=1) samples, it was not used to distinguish samples in subsequent analyses.

Compared to field-preserved lake fish, the mean telencephalon shapes of field-preserved anadromous fish from Rabbit Slough and Mud Lake had extreme negative values on PC1 and slightly positive values on PC2. Thus, their mean telencephalon shapes were convex and laterally extended, which was similar but not identical to those of field-preserved benthic populations. Along PC1, telencephalon shapes of field-preserved anadromous fish from Rabbit Slough (DFA: χ^2=59.690, p<0.001, df=1) and Mud Lake (DFA: χ^2=84.884, p<0.001, df=1) were statistically different from those of field-preserved, lake-fish samples.

The mean telencephalon shape of lab-bred anadromous fish from Rabbit Slough occupied a unique position in the morphospace, with extreme positive values of PC1, which represented concave shapes, and negative values of PC2, which represented longitudinally elongate shapes. The telencephalon shapes of lab-bred Rabbit Slough fish were statistically different from those of field-preserved Rabbit Slough specimens (DFA: χ^2=69.464, p<0.001, df=1) and of lab-bred lake fish (DFA: χ^2=66.835, p<0.001, df=1) along PC1.

Relative Telencephalon Size - Field-Preserved, Lab-Held (90 days), and Lab-Bred Samples

Differences between benthics and limnetics for relative telencephalon centroid size were tested using ANCOVA. Relative telencephalon sizes were greater in pooled benthics than in pooled limnetics in the field-preserved ($F_{1,255}$= 8.426, $p<0.01$), lab-held ($F_{1,91}$=16.599, $p<0.001$), and lab-bred ($F_{1,84}$=32.644, $p<0.001$) treatments. A detailed analysis among lake samples could reveal whether this ecotypic difference was due to the contributions by all or only a subset of samples from each ecotype. Thus, relative telencephalon sizes were compared among populations within each treatment type separately. An ANCOVA detected differences in relative telencephalon sizes among lake samples in each treatment (field-preserved, $F_{5,251}$= 16.565 $p<0.001$; lab-held, $F_{5,87}$= 6.913 $p<0.001$; lab-bred, $F_{5,80}$=11.108, $p<0.001$). A GT2-method test for unplanned comparisons was used to determine which population means differed from each other (Fig. 4). The rank order of populations remained the same across treatments, except for the position of Lynne Lake. Lynne ranked second among the field-caught fish, third in the lab-held fish, and fourth in the lab-bred fish, so that larger relative telencephalon sizes of the benthic ecotype could be explained by the contribution of all benthic samples in only the lab-bred treatment.

Data for all fish from field-preserved lake samples were pooled and compared to those of anadromous fish from Rabbit Slough or Mud Lake. The relative telencephalon sizes of field-preserved lake fish were greater than those from field-preserved anadromous fish from Rabbit Slough ($F_{1,299}$=81.249, $p<0.001$) and Mud Lake ($F_{1,284}$=140.365, $p<0.001$). Similarly, lab-bred lake samples were pooled and their relative telencephalon sizes were compared to those of lab-bred anadromous fish from Rabbit Slough. Unlike the results using field-preserved fish, lab-bred

anadromous fish had larger relative telencephalon sizes than those of lake fish ($F_{1,111}$=5.157, $p<0.05$).

Intra-population treatment effects were studied separately for each population (Fig. 5). An ANCOVA detected differences in relative telencephalon sizes among field-preserved, lab-held, and lab-bred samples for each lake population (Corcoran, $F_{2,78}$= 7.881, $p<0.001$; Mud, $F_{2,62}$= 7.002, $p<0.01$; Walby, $F_{2,70}$=4.591, $p<0.05$; Long, $F_{2,80}$= 6.580, $p<0.01$; Lynne, $F_{2,69}$=20.524, $p<0.001$; South Rolly, $F_{2,56}$=7.802, $p<0.01$). Generally, field-preserved samples appeared to have greater relative telencephalon sizes than lab-bred or lab-held samples, but the GT2-method test for unplanned comparisons indicated that these differences were not always statistically significant. Relative telencephalon sizes for field-preserved fish were greater than those of lab-held fish in all cases except for the Long Lake population. Relative telencephalon sizes for field-preserved fish were nominally greater than those of lab-bred fish, but these results were not significant for Mud and Walby lake populations. This test failed to detect a difference between relative telencephalon sizes of lab-held and lab-bred samples in any lake population.

Relative telencephalon sizes of field-preserved anadromous fish from Rabbit Slough were compared to those of lab-bred anadromous Rabbit Slough fish. An ANCOVA could not be used for this comparison because the assumption of homogeneity of slopes among samples was violated. Thus, size-adjusted telencephalon sizes were compared using predicted telencephalon sizes based on sample regressions. Linear regression equations were statistically significant for field-preserved ($F_{1,42}$=176.193, $p<0.001$) and lab-bred ($F_{1,25}$=47.321, $p<0.001$) Rabbit Slough samples. A predicted telencephalon size was calculated using the collective brain area mean (BA=17.841 mm^2) of each sample. Contrary to expectations, lab-bred anadromous fish from

Rabbit Slough had larger relative telencephalon sizes than those of field-preserved counterparts (t_{69}=7.399, p<0.001).

The Effect of Time in Captivity on Telencephalon Shape and Size (Corcoran and Long Lakes)

To study the effect of time in captivity on telencephalon morphology, lab-held fish from the Corcoran (benthic) and Long (limnetic) lake populations were sampled at 30, 60, and 90 days in captivity, and the telencephala of these fish were compared to field-preserved and lab-bred counterparts. Consistent with the general ecotypic analysis, the telencephalon shapes of field-preserved fish from Corcoran Lake were rounder than those from Long Lake (DFA: χ^2= 10.880, p<0.001, df=1; Fig. 3).

Compared to field-preserved fish, the longer that lab-held fish were held in captivity, the more their telencephalon shapes were longitudinally extended and rounder in the anterior portion (i.e., landmarks 8-12; Fig. 6). In Long Lake fish, the magnitude and direction of change in position in the morphospace of mean telencephalon shape from field-preserved fish to 30-day lab-held fish was similar to that of 30-day to 60-day lab-held fish. In Corcoran Lake, a similar shape change occurred from field preserved to 30-day lab-held fish, but the telencephalon shapes from 60-day lab-held fish were located adjacent to their 30-day counterparts. In both populations, the telencephalon shapes of 60- and 90-day lab-held fish occupied the same area in the morphospace. The telencephalon shapes of lab-bred and 90-day lab-held fish did not differ along PC1 for Corcoran (DFA: χ^2= 0.0292, p=0.86, df=1) or Long (DFA: χ^2= 0.0127, p= 0.91, df=1) lakes. Thus, compared to the telencephalon shapes of field-preserved fish, those of lab-held fish from Corcoran and Long lakes became more like the telencephala of lab-bred fish over

the course of 90 days in captivity. However, the lab-held fish from Corcoran Lake did not move as far in the morphospace during their period of captivity as the Long Lake lab-held fish did, but they reached their final position after 30 days of captivity compared to 60 days for the Long Lake fish.

Using natural-log transformed dorsal brain area as the covariate, an ANCOVA detected differences in relative telencephalon sizes across treatments for Corcoran ($F_{4,108}$=5.728, $p<0.001$) and Long ($F_{4,108}$=5.273, $p<0.001$) lake fish. In Corcoran Lake, the relative telencephalon sizes of fish from all three lab-held samples were statistically smaller than those of their field-preserved counterparts (Fig. 7). While relative telencephalon sizes of fish from all the lab-held Long Lake samples were also smaller than field-preserved ones, only the 30-day lab-held fish were statistically different (Fig. 7). Given that the overall pattern was consistent in the two populations, failure to detect a difference between field preserved, 60-day lab-held, and 90-day lab-held Long Lake fish was probably due to the lack of statistical power in the lab-held samples. The relative telencephalon sizes of lab-bred fish were similar to those of their lab-held counterparts in both populations. Thus, in Corcoran and Long lake populations, the telencephala of wild fish appeared to undergo a drastic decrease in relative size when they are held in aquaria for at least 30 days, resembling those of lab-bred counterparts, which was not followed by substantial additional reduction for up to 90 days in captivity.

DISCUSSION

Using samples from bottom-feeding (benthic), open-water planktivore (limnetic), and anadromous threespine stickleback populations, we investigated telencephalon plasticity due to experience under natural or captive conditions. As before (Park and Bell 2010), the

telencephalon shapes of field-preserved and 90-day lab-held benthics were convex and round while those of limnetics were more concave and triangular, but no such pattern was found in lab-bred fish. Benthics also had larger relative telencephalon sizes than limnetics across all treatments, and this difference was most pronounced among lab-bred samples. Among samples from each lake, field-preserved fish consistently had larger relative telencephalon sizes than lab-held and lab-bred fish. Taken together, these results are consistent with the interpretation that the size and shape of the telencephalon depends on phenotypic plasticity and that this plasticity is a general property of stickleback populations. However, greater size of the telencephalon in benthics is independent of experience.

Potential Confounding Variables

Three potential confounding variables that could explain findings in the present work are sexual dimorphism, selective mortality, and the inclusion of sexually mature fish in field-preserved samples. In stickeback, males tend to have more triangular telencephalon shapes and larger relative telencephalon sizes than females (Park & Bell 2010). The sex ratios of samples used in the current study were more-or-less similar across samples (Table 1), and therefore, sexual dimorphism is unlikely to explain the present findings.

Ecotypic differences in the telencephalon shapes of field-caught fish that are absent in lab-bred fish could have been due to sampling effects caused by selective mortality occurring in the wild (see Brown & Braithwaite 2004). Unlike lab-bred fish, field-caught fish may have been ecotypically divergent because fish that did not contribute to these differences did not survive prior to capture. Determining the effect of selective mortality poses a difficult challenge in any investigation that compares field-preserved and lab-bred fish, and other investigators who have

employed the same method to study brain plasticity did not address this issue (Marchetti & Nevitt 2003; Kihslinger *et al*., 2006). However, if phenotypic plasticity was not important in stickleback, then the telencephala of field-preserved and lab-held fish, both of which were field-caught, should have had similar characteristics, but compared to field-preserved fish, lab-held fish had telencephalon shapes that occupied a different area in the PCA (Fig. 3) and had smaller relative telencephalon sizes (Fig. 5). Thus, while the current methods were unable to provide a rigorous test of potential sampling effects due to selective mortality in natural populations, a comparison of field-preserved and lab-held fish indicates that telencephalon plasticity is characteristic of stickleback.

Sexual maturation can trigger a change in brain morphology. In cowbirds, the hippocampus grows larger during the breeding season (Clayton *et al*. 1997). In the present study, field-preserved fish had different telencephalon shapes (Fig. 3) and larger relative telencephalon sizes than lab-held and lab-bred fish (Fig. 5). These differences could be explained by the contribution of adult fish in field-preserved samples. Threespine stickleback from Cook Inlet lakes typically take two years to mature (Havens *et al.* 1984; Heins *et al.* 1999), but our lab-held and lab-bred fish were sacrificed as pre-reproductives, a classification based on age or the lack of nuptial coloration. In contrast, field-preserved fish were collected from a broad range of body sizes to compensate for potential variability due to limited sample sizes in lab-held and lab-bred samples. Field-preserved samples undoubtedly included one year-old (pre-reproductive) and two year-old (adult) fish. The larger relative telencephalon of field-preserved fish could be due to positive allometry of adult fish. However, a test for homogeneity of slopes, which is an assumption of ANCOVA, failed to detect differences in the telencephalon growth trajectories using dorsal brain area as the covariate among field-preserved, lab-bred, and lab-held samples

for each population (see Appendix 3), suggesting that differences in telencephalon morphology among fish from the different treatment types were unlikely to be due to the presence of adult fish in field-preserved samples.

Telencephalon Shape in Relation to Inferred Ecology

The dorsal shapes of the telencephala of field-preserved benthic stickleback are generally round while those of limnetics are more triangular (Park & Bell 2010). If telencephalon morphology is genetically based, ecotypic differences should be maintained between benthic and limnetic samples of lab-held and lab-bred fish. In the present work, PC1, which accounted for nearly twice the variation of PC2, captured an ecotypic pattern in field-preserved and lab-held lake samples that was consistent with findings from Park and Bell (2010). In contrast, this ecotypic pattern disappeared in lab-bred fish. The reduced shape differences in lab-held fish and the lack thereof in lab-bred fish relative to field-caught fish could be a consequence of experience in captivity. In support of this interpretation, in Corcoran and Long lakes, the telencephalon shapes of lab-held fish were more similar to those of field-preserved fish when sampled at 30 days than at 60 or 90 days in captivity (see below), and fish from samples sacrificed at 90 days have telencephalon shapes that are indistinguishable from those of lab-bred fish along PC1.

The loss of ecotypic shape differences in captive fish may reflect absence of differences in telencephalon functions that typically diverge between wild benthics and limnetics. Compared to field-caught benthics, field-caught limnetics exhibit shorter memory for handling prey (Mackney & Hughes 1995), less male-male aggression (Scotti & Foster 2007), poor spatial learning (Odling-Smee & Braithwaite 2003a; Odling-Smee *et al*. 2008; Park in preparation), and

more conspicuous courtship behavior (Foster 1994). Differences in aggression among stickleback populations are heritable (Bell 2005), but the extent to how these differences may be affected by plasticity is unknown. Similarly, while mating and parental care are mediated by the telencephalon (Pollen *et al*. 2007; see Gonzalez-Voyer *et al.* 2009), the relationship of these traits to habitat type and plasticity is poorly understood. In contrast, spatial learning varies with ecology (Sherry 1998) and depends considerably on phenotypic plasticity (Juraska *et al*. 1985, 1989; Vargas *et al*. 2000). The greater telencephalon convexity of benthics compared to limnetics may reflect greater volume of the dorsolateral region (Dl) of the telencephalon (Park and Bell 2010), which processes spatial learning in fishes (Fig.1; Salas *et al*. 1996a,b; Vargas *et al*. 2000, 2009; Rodríguez *et al*. 2002; Broglio *et al*. 2003; Northcutt 2006) (see Fig. 3). Dl is homologous to the hippocampus of tetrapods (Vargas *et al*. 2000, 2009). In birds and mammals, greater size of the hippocampus is associated with superior spatial learning (O'Keefe & Nadel 1978; Sherry 1998). Birds that use spatial learning to cache seeds and migrate have a larger hippocampus than closely related species that do not (Clayton & Krebs 1994; Healy 1996, 1998; Krebs & Davies 1997; Healy *et al*. 2005). Similarly, compared to sedentary females, male kangaroo rats (*Dipodomys*) and meadow voles (*Microtus*) have larger home-ranges, better spatial learning ability, and a larger hippocampus (Jacobs & Spencer 1994; Jacobs *et al*. 1990). Like the hippocampus, larger Dl size in the telencephalon is associated with larger home-range sizes in blennies (Carneiro *et al*. 2001) and with residence in structurally complex littoral habitat in cichlids (Shumway 2008). In stickleback, field-caught benthics which occupy more complex littoral environments have rounder telencephalon shapes (Park and Bell 2010) and superior spatial learning ability than field-caught limnetic (Odling-Smee *et al.* 2008, Park in preparation) and river (Girvan & Braithwaite 1998) stickleback, both of which have more triangular

telencephalon shapes than benthics (see Park & Bell 2010). Therefore, wild benthic stickleback may have a larger Dl that allows for better spatial learning than limnetics, and when these fish are brought into captivity, this distinction may disappear due to the lack of environmental stimulation needed to maintain the neural traits necessary for this learning ability.

An unexpected finding was that the telencephalon shape differences as a result of treatment were prominent. In going from field-preserved to lab-held or lab-bred samples, there was a shift towards more negative values along both PC axes. As with the ecotypic analysis, these results indicate that telencephalon shape is greatly affected by phenotypic plasticity. Obviously, further study of reaction norms of telencephalon shape is needed, but we speculate that if fish from one ecotype were grown in a very different habitat, their telencephalon shapes may be so strongly shifted that any genotypic effect of ecotype would be masked. Therefore, to the extent that changes to telencephalon shape influence associated behaviors, experience may be critical for their maintenance in stickleback.

Telencephalon Size in Relation to Inferred Ecology

Park and Bell (2010) studied a range of freshwater populations and did not detect a difference between field-preserved benthics and limnetics using linear brain length as the covariate. The present work included a subset of these populations, and instead of brain length, dorsal brain area was used as the covariate to standardize telencephalon centroid size. Unlike previous findings, field-caught benthics had larger relative telencephalon sizes than limnetics, but a more detailed analysis revealed that the populations with the greatest relative sizes were not exclusively benthics. A field-caught sample from Lynne Lake (limnetic) had a larger mean relative telencephalon size than those from two benthic populations and from one lab-held

sample from a benthic population. While brain area and brain length were expected to be highly correlated, it is possible that there was ecotypic variation along lateral aspects of the brain. Compared to fish species that occupy structurally complex habitat, pelagic species tend to have larger eyes which are associated with larger optic lobes (Kotrschal *et al*. 1998). In stickleback, limnetics occupy open areas of lakes and have larger eyes than benthics (reviewed in Bell & Foster 1994; McPhail 1994; Walker 1997), and therefore, limnetics may also have larger optic lobes. Thus, relatively larger optic lobes in limnetics and the exclusion of other benthic and limnetic populations could account for the inconsistency in findings between the current study and Park and Bell (2010).

Inter-population differences in relative telencephalon size may reflect the divergence of a variety of behaviors. Compared to fishes that live in open water habitat, those that live in structurally complex habitats have larger relative telencephalon sizes (Bauchot *et al*. 1977, van Staaden *et al*. 1994/95; Huber *et al*. 1997), which may be associated with enhanced spatial learning ability (see Kotrschal *et al*. 1998). Larger relative telencephalon size is also associated with greater parental care (Gonzalez-Voyer *et al.* 2009) and sociality (Huber *et al.* 1997; Kotrschal *et al.* 1998; Hofmann 2001; Pollen *et al.* 2007). Thus, fish with larger relative telencephalon sizes may be superior spatial learners, committed to greater parental care, or are more social.

In contrast to field-caught fish, the largest relative telencephalon sizes among lab-bred fish belonged exclusively to benthics. This result can be explained by the change in relative rank of Lynne Lake fish, which fell from second highest among field-preserved samples to fourth highest in the lab-bred treatment (Fig. 4). Lab-bred fish were raised under common garden conditions, and therefore, these results suggest that benthic-limnetic differences in relative

telencephalon size could be heritable and that the Lynne Lake population is especially sensitive to environmental stimulation. While we acknowledge that these results are preliminary, investigation into the possibility of inter-population variation for norms of reaction of telencephalon plasticity, as mentioned earlier, is worth further study. Nonetheless, the general pattern of population differences using field-preserved fish differed from that using lab-bred fish. Thus, phenotypic plasticity may have a large impact on our perception of differences among populations depending on how samples are acquired. For example, in ninespine stickleback, lab-bred marine fish had larger relative telencephalon sizes than lab-bred pond fish, indicating heritable differences among populations (Gonda *et al.* 2009a), but our findings suggest that there is great potential for such results to be compromised by the effects of phenotypic plasticity in the wild.

In each population, field-preserved fish consistently had larger relative telencephalon sizes than fish held in the lab for 90 days or bred in the lab. Two possible explanations for these differences could be environmental stimulation or varying growth rates of different brain structures during ontogeny (see Kihslinger *et al.* 2006). Experiences in the wild may induce growth of the telencephalon in wild fish. In mammals, environmental enrichment induces cell proliferation and dendritic growth in the hippocampus (Juraska *et al.* 1985, 1989; van Praag *et al*. 2000; Rampon *et al.* 2000; Faherty *et al*. 2003). In birds, experience with storage and retrieval of food triggers the increase of neuronal number and overall brain volume (Patel *et al*. 1997). In fishes, experience with spatial tasks induces protein synthesis in the dorsolateral region of the telencephalon (Vargas *et al.* 2000). Thus, under natural conditions, a variety of environmental stimuli associated with enhanced spatial learning could stimulate positive neurological changes in the telencephalon of stickleback.

Alternatively, limited environmental stimuli in captivity could have caused shrinkage of the telencephalon in lab-held fish. In mammals, lack of maternal contact early in life resulted in the production of immature neurons and decreased cell proliferation in offspring (Mirescu *et al.* 2004). Nest-searching cowbirds that were prevented from locating host nests had smaller relative hippocampus sizes than individuals with access to nests (Day *et al.* 2008). Elevated stress from starvation or corticosterone administration decreased the size of adult brain nuclei in birds (Buchanan *et al.* 2004). Similarly, stress induced by elevated temperatures caused reduction of neuronal cell number in pejerrey fish (*Odontesthes bonariensis*; Miranda *et al.* 2003). Social factors can also cause loss of neural tissue. Greater exposure to aggression reduced neuron size in pup fishes (*Cyprinodon nevadensis*; Lema 2006). In jewel fish (*Hemichromis bimuculatus),* crowding induced the loss of dendritic spines in neurons (Burgess & Coss 1982), and in ninespine stickleback (*Pungitius pungitius*), pond fish bred in a group had smaller overall brain sizes than fish raised individually (Gonda *et al.* 2009b). Therefore, lab-bred and lab-held stickleback from the current study may have had smaller relative telencephalon sizes than wild counterparts because they were deprived of relevant environmental stimulation.

Another possible explanation for differences in relative telencephalon size between field-preserved and lab-bred fish could be that different parts of the brain grow at dissimilar rates under natural versus captive conditions, potentially biasing calculations of relative telencephalon size brain area. Salmon undergo a drastic loss in body weight (but not body length) during smoltification which may bias estimates of relative telencephalon sizes based on body weight (Pankhurst & Montgomery 1994; Kihslinger *et al*. 2006). By analogy, the telencephalon of lab-bred fish may not grow as fast as other parts of the brain (e.g., optic lobes, cerebellum) due to a number of factors such as diet or environmental stimulation. Thus, smaller relative telencephalon

size in lab-bred fish compared to field-preserved fish may be an outcome of faster growth of brain structures other than the telencephalon. The possibility of variable growth rates of different brain parts in fishes is not well known, and further study is needed to understand its importance in this and other species.

The Effect of Time in Captivity on Telencephalon Morphology

The influence of time in captivity on telencephalon morphology was ascertained in the Corcoran (benthic) and Long (limnetic) lake populations by comparing field-preserved fish to lab-held fish maintained in captivity for 30, 60, and 90 days. Compared to field-preserved fish, lab-held fish collected at 30 days underwent a dramatic reduction in relative telencephalon size, with minimal subsequent change occurring at 60 and 90 days. In contrast, telencephalon shapes changed gradually over the same time period, with lab-held counterparts becoming more and more elongate but laterally rounder in the anterior portion of the telencephalon (i.e., landmarks 8-12), yielding an overall shape that resembled those of lab-bred fish. Given that relative telencephalon sizes were largest in field-preserved fish, it is possible that the rounder anterior features of telencephalon shapes of 90-day lab-held fish could be due to the loss of neural tissue in areas more posterior. However, the change of shape cannot only be due to loss of tissue in some places since the shape continued to change after the size became stable. Thus, the reasons underlying change in telencephalon shapes of lab-held fish relative to field-caught counterparts remain unclear. However, the contrast between an abrupt change of telencephalon sizes and a gradual change of telencephalon shapes in lab-held fish suggests that some parts of the telencephalon may have responded sooner than others while these fish were in captivity. A decrease in relative brain size may reflect loss of a variety of functions already mentioned. In

brief, in fishes, stressors such as elevated temperatures (Miranda *et al.* 2003) and crowding (Burgess & Coss 1982; Gonda *et al.* 2010b) during captive care are associated with neurological deficits. The relationship between time in captivity and its effect on subregions within the telencephalon has never been studied in fishes. It is also unknown whether the acquisition or loss of neural tissue in the telencephalon occurs gradually or during critical periods (see Thorpe 1958; Penfield & Roberts 1959; Almli & Finger 1987).

The Ancestral Condition for Phenotypic Plasticity of the Telencephalon

Anadromous stickleback can be used to infer the ancestral condition of traits that are found in derived freshwater populations (Bell 1976, 1995). Anadromous fish feed on plankton at sea (Mackney & Hughes 1995) and have trophic morphology resembling that of limnetics (McPhail 1994). In the field, anadromous stickleback can easily be distinguished from derived lake fish based on adult body size. Generally, threespine stickleback from Cook Inlet typically achieve reproductive condition in two years (Havens *et al.* 1984; Heins *et al.* 1999) with lake and anadromous fish achieving standard lengths of 35-50 mm and 60-70 mm, respectively (see Table 1).

Compared to other field-preserved samples, anadromous samples had more negative values for telencephalon shapes on PC1 (Fig. 3), which corresponds to very convex shapes resembling but not identical to those of benthics. This trait may be the ancestral condition that was retained in benthic populations (see Park & Bell 2010). Curiously, the mean telencephalon shape of lab-bred anadromous fish from Rabbit Slough occupied a unique area in the morphospace. Compared to those of field-preserved anadromous fish, lab-bred anadromous fish possessed telencephala that were considerably more triangular (positive PC1) and elongate

(negative PC2), and for reasons already mentioned, this dorsal telencephalon morphology is likely a consequence of being bred under captive conditions.

Field-preserved anadromous fish had smaller relative telencephalon sizes than lake fish, which is consistent with findings by Park and Bell (2010). Contrary to expectation, lab-bred anadromous fish had larger relative telencephalon sizes than field-preserved counterparts and lab-bred lake fish. While these results seem paradoxical, they are consistent with findings from ninespine stickleback (*Pungitius pungitius*) showing that laboratory-bred ancestral, marine populations had larger mean relative telencephalon sizes than pond populations (Gonda *et al.* 2009a). One possible explanation for the results in threespine stickleback could be that smaller relative telencephalon sizes in field-preserved anadromous fish reflect plastic responses that are a consequence of migration. Field-preserved anadromous stickleback are migratory and semelparous, and it is well-known that anadromous salmon undergo immense muscular and physiological degradation while returning to breeding sites (Ando *et al.* 1986). Thus, the larger mean relative telencephalon sizes of lab-bred anadromous fish may reflect the lack of neurological atrophy that results from migrating back from the ocean to freshwater breeding grounds.

In the present study, lab-bred anadromous fish were sampled at comparable body sizes to those of lake fish (i.e., 35-50 mm), but field-preserved anadromous samples consisted exclusively of larger (i.e., 60-70 mm) two-year old anadromous adults (see Table 1). In Rabbit Slough, mature one-year old anadromous fish that are similar in body size to two-year old lake adults occasionally occur. If relative telencephalon sizes of lab-bred anadromous fish are still larger than those of their one-year old field-preserved counterparts, then phenotypic plasticity in the form of neurological atrophy due to migration would be supported. On the other hand, if

relative telencephalon sizes do not differ between lab-bred and field-preserved anadromous fish of comparable body sizes, then smaller relative telencephalon size may be characteristic of the larger wild two-year old anadromous fish. One interpretation for this possibility is that evolution of smaller body size in freshwater fish resulted in larger relative brain size because there is negative growth allometry of the brain in anadromous fish (Davis & Miller 1967). It is also possible that body size becomes ontogenetically decoupled from absolute brain size in the two-year old anadromous fish (see Striedter 2005). During development, absolute telencephalon size may increase until a particular body size is achieved, after which only body size continues to increase. In mammals, the body and brain grow at similar rates early in ontogeny, but as individuals mature, brain size remains roughly constant while body size continues to increase (Count 1947). A similar process may occur in two-year old anadromous stickleback because the brain is metabolically expensive (Dukas 1999), and additional neural tissue may be not be necessary to carry out the basic functions of these larger stickleback. Unfortunately, the methods used in the present study cannot distinguish between these two possibilities, and further research on allometric brain growth in anadromous threespine stickleback is needed.

Phenotypic Plasticity at the Interface of Brain and Behavior

Phenotypic plasticity affects numerous traits in threespine stickleback (Lindsey 1962; Wund *et al.* 2008) and may be demonstrably adaptive (Swain 1992). Environmental effects may be particularly important for the telencephalon. Wild, field-preserved stickleback consistently had larger relative telencephalon sizes than lab-held and lab-bred fish, and ecotypic telencephalon shape differences disappeared in lab-bred fish. These findings are consistent with reports from a variety of other vertebrate taxa. Hatchery-bred rainbow trout (Marchetti & Nevitt

2003; Kihslinger *et al*. 2006) and lab-bred guppies (Burns *et al.* 2009) had smaller relative telencephalon sizes than those caught in the field. Domesticated birds and mammals have smaller relative brain sizes than their wild counterparts (Ebinger & Rohrs 1995; Kruska 1988). Compared to limnetics, plasticity may be more important in benthic stickleback, which have larger, rounder telencephala, because they have a higher upper range for importance of spatial learning (Park unpublished data; also see Odling-Smee *et al.* 2008). In support of this contention, cowbirds held in captivity had smaller relative hippocampus sizes than field-caught counterparts presumably due to limited spatial learning (Day *et al.* 2008). The importance of telencephalon plasticity remains unclear in anadromous stickleback. Ancestral marine stickleback exhibit phenotypic plasticity for behaviors mediated by the telencephalon (see Shaw *et al.* 2007), and thus, telencephalon plasticity should be substantial in anadromous fish.

Future research should aim to understand brain plasticity in relation to behavior. While the evolution of behavioral plasticity is an active topic of research (see West Eberhard 2003; Pigliucci & Murren 2003; Price *et al*. 2003; Shaw *et al*. 2007), its relationship to brain plasticity is virtually unknown. Behavioral change may precede morphological change in evolution because behavior is usually more plastic, and thus, behavior is more likely to respond sooner in a new environment, imposing new demands that are met with morphological change (Baldwin 1896, 1902; Rau 1933; West-Eberhard 1989; Wcislo 1989). For example, behavioral plasticity in the expression of mating behaviors (e.g., zigzag dance) in the threespine stickleback is present in ancestral populations, which may have influenced evolutionary differences among derived freshwater populations (Shaw *et al*. 2007). Similarly, corresponding changes to telencephalon morphology may occur in these populations after demands for spatial learning have already been imposed. However, it is also possible that if morphological changes occur first, this would

inevitably be followed by a change in affected behaviors (Romer 1958; Colbert 1958). For example, any negative impact on the neurology of fishes, as often occurs in captive care (see Burgess & Coss 1982, Miranda *et al*. 2003, Mirescu *et al*. 2004, Buchanan *et al*. 2004), may impact the evolution of associated behaviors. On the other hand, one could argue that the plasticity of the telencephalon that we and others report (e.g., Marchetti & Nevitt 2003; Kihslinger *et al.* 2006; Burns *et al*. 2009) only buffers against evolutionary change (see Sulton 1992; Schlicting 1986). Thus, it is clear that many critical questions about the relationship between brain plasticity and the evolution of brain and behavior remain unanswered, but the threespine stickleback species complex provides a powerful neurobiological, behavioral, and evolutionary model system to further explore such research questions.

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Table 1. Sources and composition of threespine stickleback samples. Samples include: FP, field-preserved; LH_{30}, 30-day lab-held; LH_{60}, 60-day lab-held; LH_{90}, 90-day lab-held, and LB, lab-bred. Symbols are as follows: Year, collection year; n, sample size by sex; SL, standard length; BS, body centroid size, and SE, standard error (Fig. 3).

Population	Ecotype	Sample	Year	$n_{♂}$	$n_{♀}$	Mean SL ± SE	Mean BS ± SE
Corcoran Lake	Benthic	FP	2007	26	23	42.353 ± 0.854	51.535 ± 1.062
		LH_{30}	2007	6	11	37.682 ± 0.413	47.148 ± 0.521
		LH_{60}	2007	6	9	40.461 ± 0.468	50.279 ± 0.580
		LH_{90}	2007	9	6	39.370 ± 0.586	48.786 ± 0.680
		LB	2008	10	8	43.928 ± 0.937	53.938 ± 1.140
Mud Lake (resident)	Benthic	FP	2008	17	20	41.647 ± 1.187	51.464 ± 1.573
		LH_{90}	2008	7	7	36.886 ± 0.867	47.493 ± 1.075
		LB	2009	9	6	39.327 ± 0.670	49.003 ± 0.869
Walby Lake	Benthic	FP	2007	28	15	43.568 ± 0.818	53.158 ± 1.027
		LH_{90}	2006	5	11	45.946 ± 0.710	56.437 ± 0.884
		LB	2008	9	6	39.245 ± 0.665	49.605 ± 0.724
Long Lake	Limnetic	FP	2007	29	26	42.393 ± 0.872	51.687 ± 1.116
		LH_{30}	2007	5	10	41.333 ± 0.553	51.418 ± 0.689
		LH_{60}	2007	11	4	40.378 ± 0.894	50.216 ± 1.186
		LH_{90}	2007	7	8	40.065 ± 0.886	49.308 ± 1.143
		LB	2008	5	9	45.189 ± 1.344	56.245 ± 1.738
Lynne Lake	Limnetic	FP	2007	32	10	45.259 ± 0.723	54.116 ± 0.916
		LH_{90}	2006	10	6	36.840 ± 1.210	43.996 ± 1.427
		LB	2008	9	6	41.863 ± 1.254	51.843 ± 1.649

South Rolly Lake	Limnetic	FP	2008	22	10	47.370 ± 1.102	58.358 ± 1.308
		LH_{90}	2008	12	6	40.987 ± 0.860	51.590 ± 0.985
		LB	2009	5	5	43.733 ± 0.666	54.146 ± 0.906
Rabbit Slough	Anadromous	FP	2003/7	30	14	68.002 ± 0.461	84.224 ± 0.677
		LB	2007	15	12	46.613 ± 0.620	57.057 ± 0.779
Mud Lake	Anadromous	FP	2003	14	15	68.877 ± 0.564	86.293 ± 0.786

Table 2. Correlation between brain size and body size in field-preserved samples. Both variables were natural log-transformed. Symbols include: n, sample size; r, Product-moment correlation coefficient; df, degrees of freedom and p, statistical p-value.

Population	**Ecotype**	**n**	**r**	**df**	**p**
Corcoran Lake	Benthic	49	0.940	47	1.165×10^{-23}
Mud Lake	Benthic	37	0.962	35	2.971×10^{-21}
Walby Lake	Benthic	43	0.891	41	1.154×10^{-15}
Long Lake	Limnetic	55	0.953	53	4.207×10^{-29}
Lynne Lake	Limnetic	42	0.742	40	1.836×10^{-8}
South Rolly Lake	Limnetic	32	0.895	30	5.180×10^{-12}
Rabbit Slough	Anadromous	44	-0.221	42	0.149 *ns*
Mud Lake	Anadromous	29	-0.084	27	0.666 *ns*

Figure 1. The threespine stickleback brain. (a) Dorsal view (anterior is up) of head with brain. From rostral to caudal, olfactory nerves, olfactory bulbs (olf), forebrain (fb), pineal gland (pg), optic lobe (opt), cerebellum (cb) and hindbrain (hb). Ventral structures (pituitary gland and hypothalamus) are not shown. (b) Transverse view (dorsal is up). Forty micron cross-section of telencephalon (Nissl-stained) at section S in part a. The three major teleost pallial subregions are the dorsomedial (Dm), dorsodorsal (Dd) and dorsolateral (Dl). Endorhinal fissure (ef) and ventral edge of sulcus limitans telencephali (slt) are shown. (c) Dorsal view (anterior is left) of the telencephalon showing fixed (1, 14) and semi-landmarks (open circles) used in the current study. Figure from Park and Bell (2010).

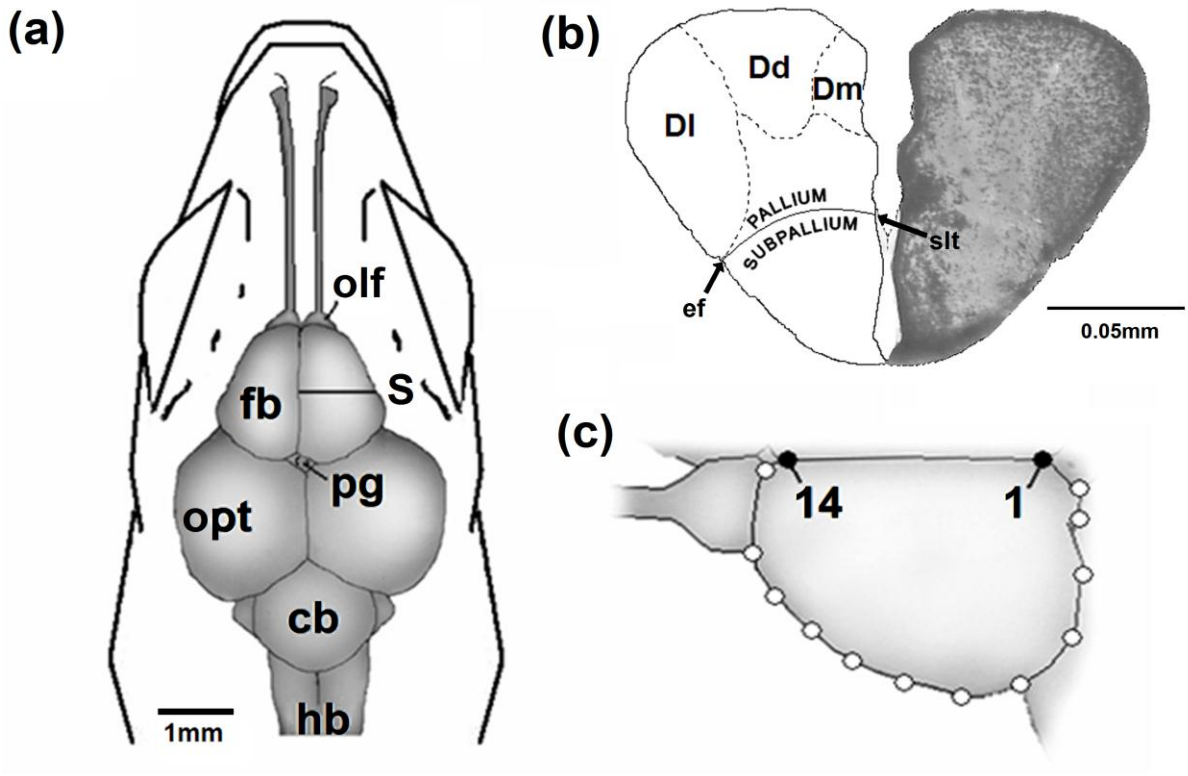


Figure 2. Potential standardizing variables. (a) Lateral view (anterior is left) of the body showing fixed landmarks. Figure adapted from Aguirre 2009. (b) Dorsal view (anterior is left) of the brain showing the area (white) used to size standardize the telencephalon.

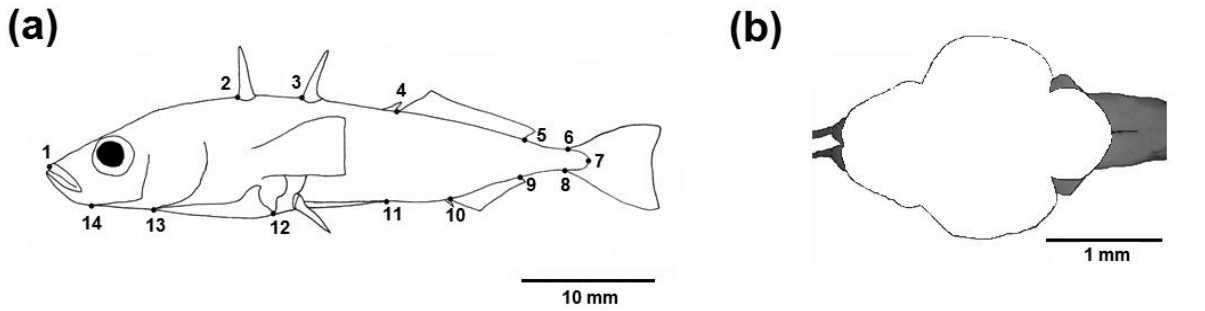


Figure 3. Principal components analysis plot showing the first two major axes of telencephalon dorsal shape variation (all populations). Points are means for each sample for field-preserved (FP), lab-bred (LB), or 90-day lab-held (LH_{90}) samples. Each set of samples in a treatment is enclosed within a shaded oval. Deformation grids describe the shapes at the ends of each axis. The symbols for ecotypes are in the inset legend. Lake sites: 1, Long; 2, Lynne; 3, South Rolly; 4, Corcoran; 5, Mud (resident); 6, Walby; 7, Rabbit Slough; 8 Mud (anadromous).

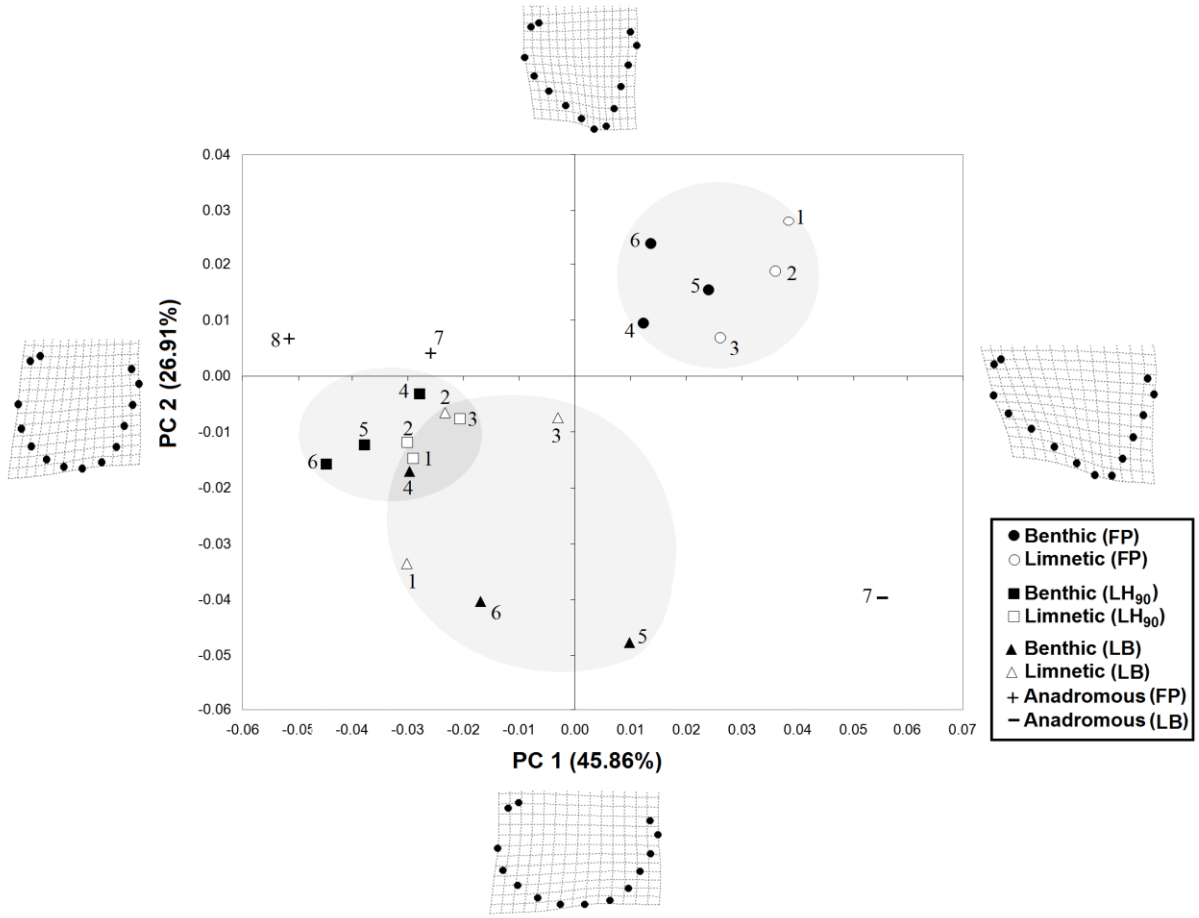


Figure 4. Inter-population comparison of relative telencephalon size. Ninety-five percent comparison limits for size-adjusted telencephalon centroid size (using ANCOVA) were calculated for each sample and compared using a GT2-method test for unplanned comparisons. Samples were compared within field-preserved, 90-day lab-held, or lab-bred treatments. All data were natural log-transformed. The symbols for ecotypes are in the inset legend of the first plot. Lake sites: 1, Long; 2, Lynne; 3, South Rolly; 4, Corcoran; 5, Mud; 6, Walby.

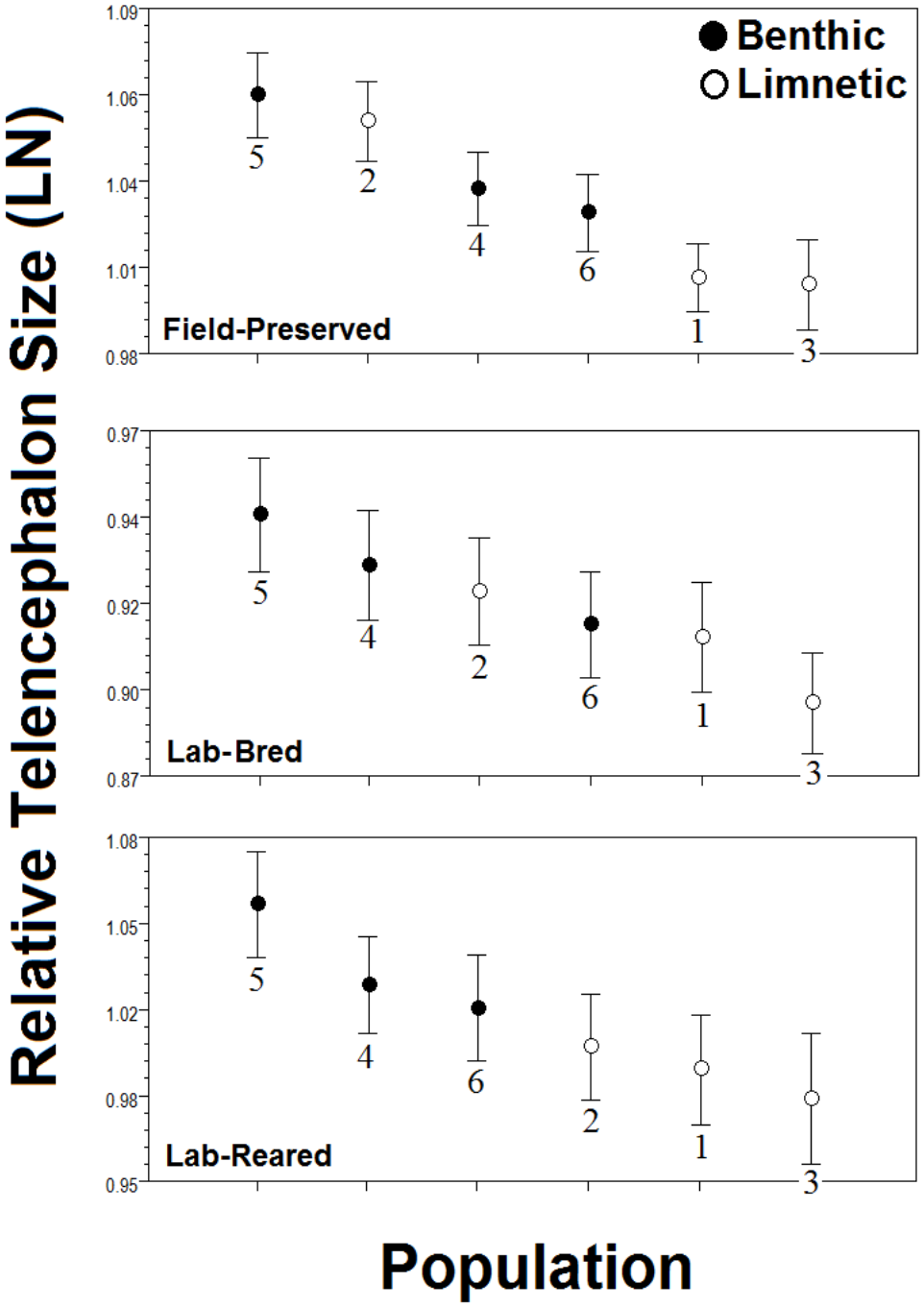


Figure 5. Intra-population comparison of relative telencephalon centroid size. Ninety-five percent comparison limits from the GT2-method test for unplanned comparisons of size-adjusted telencephalon centroid size. The name of each lake site is in its corresponding plot. All data were natural log-transformed. Samples: FP, field-preserved; LH_{90}, 90-day lab-held, and LB, lab-bred.

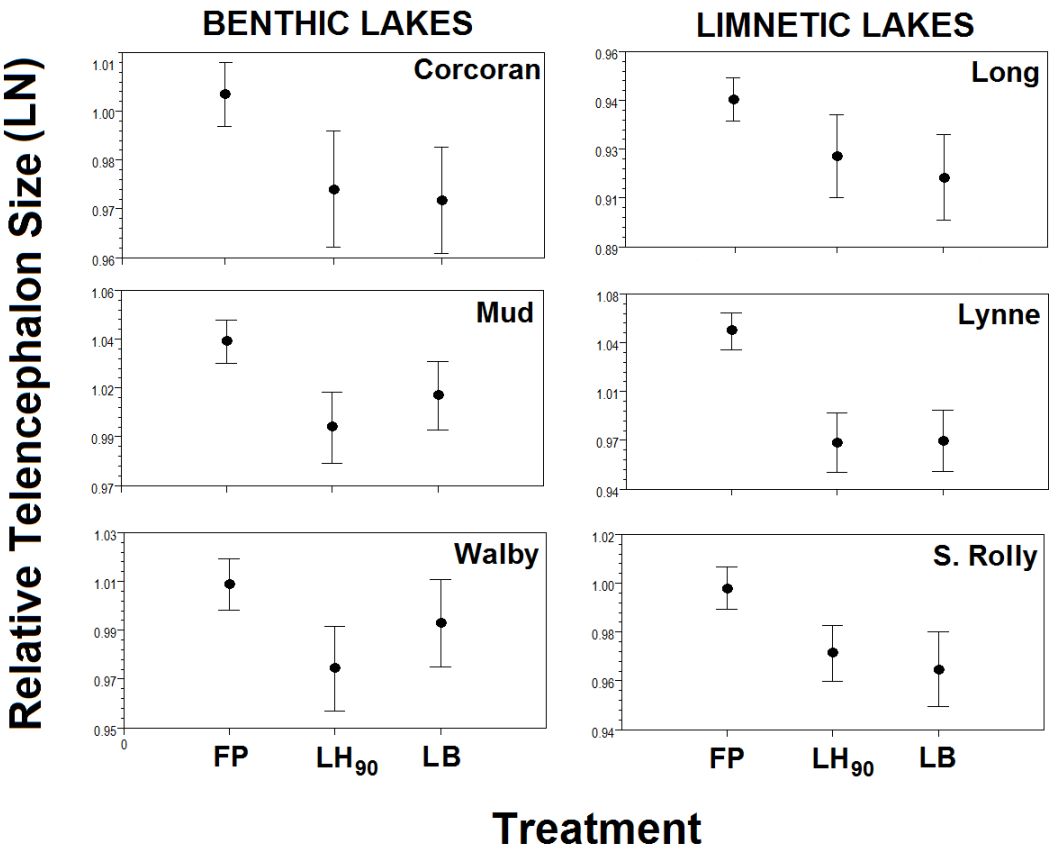
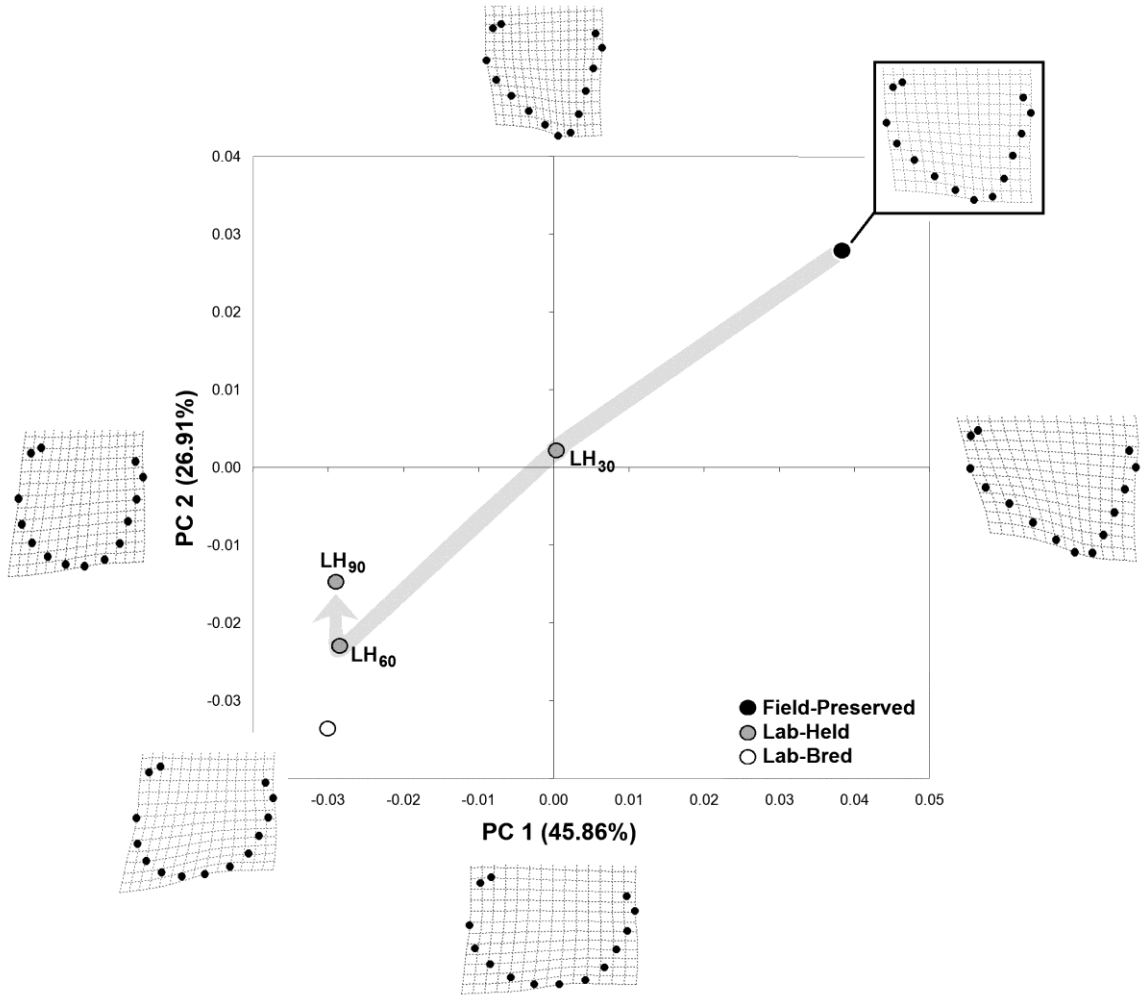


Figure 6. Principal components analysis plot showing the first two major axes of telencephalon dorsal shape variation for (a) Long (limnetic) and (b) Corcoran (benthic) lake samples. Arrow is directed from the field-preserved sample to lab-held samples kept for 30 (LH_{30}), 60 (LH_{60}), and 90 days (LH_{90}). Deformation grids describe the shapes at the ends of each axis and at the corner of negative PC1 and PC2. The mean shape of each field-preserved sample is given in a box within its plot. The symbols for the different treatments are in the inset legend. Plots (a) and (b) are a common morphospace.

(a)



(b)

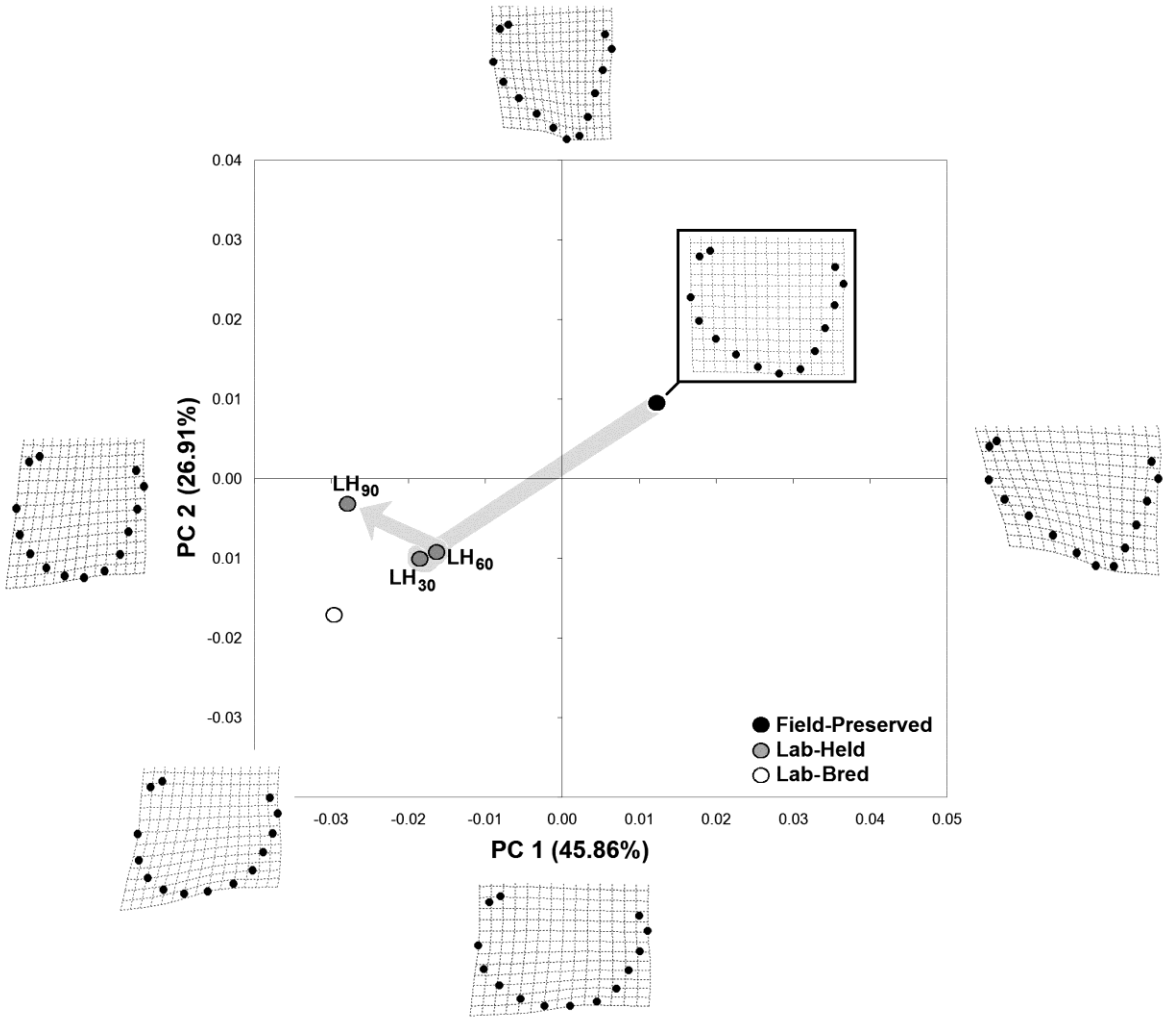
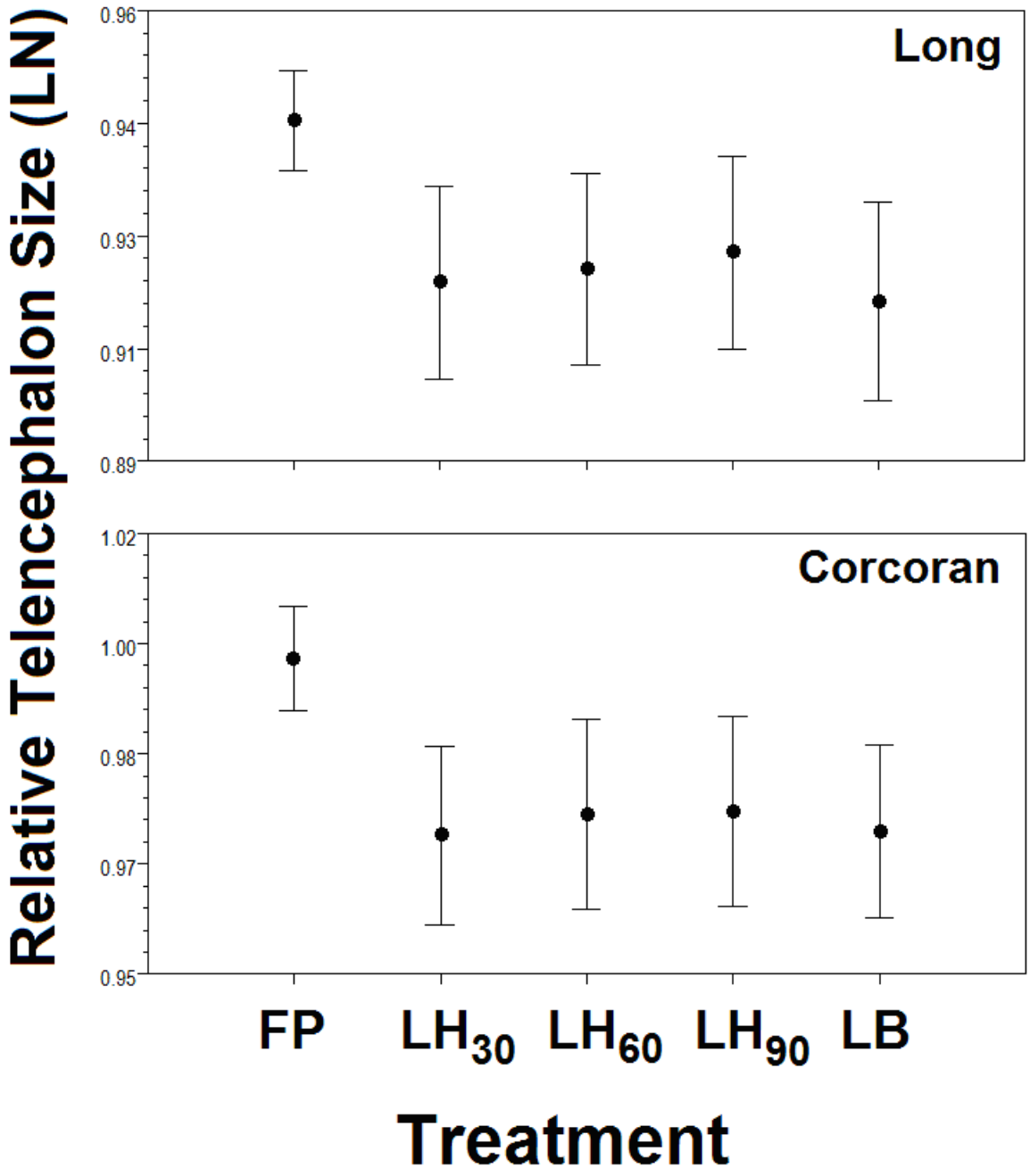

0.04
0.03
0.02
0.01
0.00
0.01
-0.02
-0.03
PC 2 (26.91%)
LH90
LH60
LH30
Field-Preserved
Lab-Held
Lab-Bred
-0.03
-0.02
-0.01
0.00
0.01
0.02
0.03
0.04
0.05
PC 1 (45.86%)

Figure 7. Analyses of relative telencephalon centroid size in Long and Corcoran lake samples. Ninety-five percent comparison limits for size-adjusted telencephalon centroid size were calculated for each sample and compared using a GT2-method test for unplanned comparisons. All data were natural log-transformed. Samples were field-preserved (FP), 30-day lab-held (LH_{30}), 60-day lab-held (LH_{60}), 90-day lab-held (LH_{90}), or lab-bred (LB). The name of each lake site is in its corresponding plot.



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Appendix 1

Attribution of Effort: Two of my dissertation chapters were co-authored by colleagues. Below I list them and their contributions to my research. I designed and wrote all chapters, and I performed all data analyses.

Chapter 3: This chapter is coauthored with Michael A. Bell. He and I collected the stickleback samples included in this study. I collected all data and performed all statistical analyses.

Chapter 4: This chapter is coauthored with Ivan Chase and Michael A. Bell. Chase provided guidance with the experimental approach. Bell and I collected all of the samples. I collected all data and performed all statistical analyses.

Appendix 2

Online Supporting Information for Chapter 3. Results from studies that validate use of left telencephalon centroid size as a proxy for telencephalon volume.

Four issues had to be addressed to validate the use of the left telencephalon centroid size as a biologically meaningful measure of differences in the *in vivo* volume of the telencephalon among populations. The first was the relationship between telencephalon centroid size and telencephalon volume. Pollen *et al.* (2007) found that volumes of cichlid brain lobes estimated based on an ellipsoid model were significantly correlated with volumes approximated from histological sections, and Burns *et al.* (2009) found that the area of the telencephalon calculated simply from dorsal images was a good proxy for telencephalon volume in teleosts. However, we are the first to use centroid size based on sliding landmarks as our estimate for volume. We could not weigh the *G. aculeatus* telencephalon to estimate size because it was difficult to detach it reproducibly from neighboring brain structures. To validate telencephalon centroid size as a proxy for *G. aculeatus* telencephalon volume, we estimated the telencephalon volume in field-caught fish using serial sections from one benthic (Corcoran Lake, n=10) and one generalist (Long Lake, n=8) lake. Specimens were fixed in 10% formalin in 0.1M PBS. Each brain was dehydrated sequentially in the following baths for 15 min each: 50%, 70%, 95% ethanol, 0.05% Eosin in 95% ethanol, 100% ethanol (3X), xylene (2X), Paraffin wax (3X, 57°C), and then submerged in a final Paraffin bath to cool and solidify.

A microtome was used to cut 12μm sections that were mounted on glass slides. Prior to staining, slides were hydrated sequentially in the following baths for 5 min each: xylene (2X), 100% ethanol (2X), 95% ethanol, 70% ethanol, 50% ethanol, and distilled water. Sections were then stained in 1% thionine (Heimer & Robards, 1981) for approximately 2 min and dehydrated using the following baths of 5 min each: distilled water, 50% ethanol, 70% ethanol, 70% ethanol with acetic acid (de-staining, < 1 min), 90% ethanol, 100% ethanol (2X), and xylene (2X). Sections were permanently mounted using Permount and a coverslip.

Digital images of all sections were taken under 10X objective with a Nikon Coolpix 995 digital camera mounted on a Zeiss Axioskop. SigmaScan Pro Image Analysis Software version 4.01.003 (SPSS Inc. 1987-1997) was used to trace the area of the left telencephalon lobe sections. The boundary of the telencephalon was traced mediolaterally around the pallium beginning at the center of the sulcus limitans telencephali to the ventral-most edge of the endorhinal fissure (Fig. 1B), as defined in Nieuwenhuys (1959). The area of each section was multiplied by its thickness (12 μm) and summed among sections.

Telencephalon centroid size and volume were natural log-transformed and used to calculate product-moment correlation coefficients in each sample with Biomstat 3.30o. Telencephalon centroid size and volume were highly correlated within Corcoran (n=10, r= 0.969, $p<0.05$) and Long (n=8, r= 0.943, $p<0.05$) lake samples. ANCOVA of natural log-transformed telencephalon centroid size using natural log-transformed volume as the covariate verified that their regression equations were not statistically different ($F_{1,15}=0.004$, $p=0.95$), indicating that differences in depth of the telencephalon between samples was minimal. Thus, centroid size is a suitable proxy for brain volume and was used in our study.

A second issue is that larger telencephalon size may not indicate more or larger neurons. The sections used in the previous validation procedure provided adequate resolution to estimate neuron number but not the size of individual neurons. Six transects, together spanning all pallial

and subpallial regions, were used to estimate the number of neurons in the median telencephalon section for each specimen. Poorly stained telencephala were excluded from this analysis. Transects were drawn between the following edges and were placed to avoid ambiguous boundaries between regions (Fig. 1B; see also Nieuwenhuys, 1959): most ventromedian edge to most dorsal edge of the telencephalon; dorsal edge of sulcus limitans to most ventrolateral edge of subpallium; dorsal edge of sulcus limitans to most lateral edge of telencephalon; most dorsomedian edge of telencephalon to endorhinal fissure; most dorsal edge of telencephalon to the edge midway between the most lateral edge of the telencephalon and the endorhinal fissure; and most ventromedian edge of the telencephalon to the edge midway between the most dorsal and lateral edges of the telencephalon. TpsDig version 2.05 (Rohlf, 2006) was used to measure transect lengths. Natural log-transformed telencephalon centroid size and natural log-transformed sum of transect lengths for each specimen were highly correlated within both the Corcoran ($n=8$, $r=0.967$, $p<0.05$) and within the Long ($n=8$, $r=0.880$, $p<0.05$) lake samples. Thus, estimate of neuron number using linear transects was used as a proxy for neuron number in the telencephalon.

All neuron cell bodies that crossed a transect were counted. The correlation between total neuron number and natural log-transformed sum of transect lengths was not significant for Corcoran ($n=8$, $r=0.595$, $p=0.12$) or Long ($n=8$, $r=0.651$, $p=0.08$) lake samples. However, these results are suggestive of a positive association between the variables, because p-values were low despite very limited samples sizes, sample correlation coefficients were similar in magnitude and direction, and when data from both samples were pooled, the correlation was statistically significant ($n=16$, $r=0.647$, $p<0.05$). Therefore, in threespine stickleback, neuron number may be positively associated with relative telencephalon size.

A third issue was the possibility that there is a systematic bias between the size of the left and right telencephalon lobes. Using existing images, centroid sizes of right telencephalon lobes were acquired for two generalist (Matanuska and Lynne) and two benthic (Mud and Corcoran) lake samples. We calculated the regression equation for natural log-transformed left telencephalon centroid size on natural log-transformed right telencephalon centroid size for each sample and tested for a significant deviation from a slope equal to one and Y-intercept equal to zero. For each regression, 95% confidence intervals for the slope included one and for the Y-intercept included zero (Matanuska, $F_{1,35}=151.470$, $p<0.05$, $Y=0.957$ (± 0.158) $X + 0.115$ (± 0.564); Lynne, $F_{1,22}=464.930$, $p<0.05$, $Y=1.032$ (± 0.099) $X - 0.021$ (± 0.302); Mud, $F_{1,57}=921.786$, $p<0.05$, $Y=0.979$ (± 0.065) $X + 0.055$ (± 0.211); Corcoran, $F_{1,17}=690.811$, $p<0.05$, $Y=1.026$ (± 0.082) $X - 0.116$ (± 0.229)). DFA did not detect shape differences between left and right telencephalon lobes (Matanuska, $\chi^2=0.188$, $p=0.66$; Lynne, $\chi^2=0.041$, $p=0.84$; Mud, $\chi^2=0.285$, $p=0.59$; Corcoran, $\chi^2=0.248$, $p=0.62$). Therefore, the use of left telencephalon centroid size and shape to infer differences between ecotypes, populations, and sexes is sufficient.

A fourth possible issue is that differences in the time brains were stored in formalin between brain extraction and image capture could affect results. 15 brains from Mud Lake (preserved using method A, extracted July 2004) and 14 brains from Willow Lake (preserved using method B, extracted Dec 2003) that were originally analyzed in Aug 2005 and Feb 2004, respectively, were photographed and digitized again in Dec 2009. The regression equation for natural log-transformed left telencephalon centroid size using earlier images on the same measures using Dec 2009 images did not deviate significantly from a slope of one and Y-intercept of zero for Mud ($F_{1,13}=68.241$, $p<0.05$, $Y=0.927$ (± 0.239) $X + 0.312$ (± 0.795)) and Willow ($F_{1,12}=99.135$, $p<0.05$, $Y=1.062$ (± 0.229) $X - 0.262$ (± 0.653)) lake samples. DFA did not

detect differences in telencephalon shapes between early and later images either (Mud χ^2=1.006, p=0.32; Willow, χ^2=3.018, p=0.08). Thus, the time interval between extraction and image capture does not seem to contribute significant measurement error to telencephalon centroid size or shape.

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Appendix 3

Online Supporting Information for Chapter 4. Supplementary work that support findings in the main study.

Validation of dorsal brain area as a proxy for overall brain size

The dorsal area of the brain used to size-standardize the telencephalon (hereafter referred to as "dorsal TOC area") included the telencephalon lobes, optic lobes, and cerebellum. To validate that dorsal TOC area was a good proxy for *G. aculeatus* overall brain size, the pattern of covariation between these variables was compared among field-preserved lake samples (Corcoran, N=35; Mud, N=23; Walby, N=20; Long, N=22; Lynne, N=18; South Rolly, N=17). Digital photographs of the whole brain from dorsal and left lateral aspect were taken using a computer-assisted video image analysis system, Sony DXC-390 video camera, mounted on a Leica MZ75 microscope. Images were acquired using ImagePro software (version 4.1.0.0 for Windows 95/NT/98). To measure left lateral whole brain area, SigmaScan Pro Image Analysis Software version 4.01.003 (SPSS Inc. 1987-1997) was used to trace the lateral outline surrounding the left olfactory bulb, telencephalon lobe, optic lobe, and hypothalamus lobe and the left aspect of the cerebellum. Dorsal TOC area included the dorsal outline surrounding the paired telencephalon and optic lobes and the single cerebellum. All area measurements were natural log-transformed. Biomstat version 3.30q (see Sokal & Rohlf, 1995) was used to carry out statistical analyses. The linear regression of dorsal TOC area on left lateral whole brain area was statistically significant for each lake population. An ANCOVA did not detect differences among lake-sample regression equations ($F_{5,128}$=1.50, p=0.194), indicating that the pattern of covariation between the two neuromorphological variables did not differ among populations. Thus, our measurement of dorsal brain area was a suitable proxy for overall brain size.

Test for positive allometry of the telencephalon in field-preserved fish

In each population, field-preserved fish had larger relative telencephalon sizes than lab-held and lab-bred counterparts. Lab-held and lab-bred samples consisted mostly of pre-reproductives, a classification based on age or the lack of nuptial coloration. In contrast, field-preserved fish included both adults and pre-reproductives. Positive allometry for telencephalon size in adult fish could explain the larger relative telencephalon sizes in field-preserved samples. To test this possibility, the allometric relationship of telencephalon size relative to brain size of field-preserved fish was compared to those of lab-held and lab-bred fish. All specimens from lake populations in the main work were used for this study (see Table 1 of main text for sample sizes), and each lake population was analyzed separately. Biomstat version 3.30q (see Sokal & Rohlf, 1995) was used to carry out all statistical tests. The linear regression equation of natural log-transformed telencephalon centroid size on natural log-transformed dorsal TOC area was statistically significant for field-preserved, lab-held, and lab-bred samples in every lake population. A test for homogeneity of slopes was used to detect potential allometric differences among samples. The slopes among field-preserved, lab-held, or lab-bred fish did not differ from one another in any lake population (Corcoran, $F_{2,76}$= 0.299, p=0.742; Mud, $F_{2,60}$= 0.092, p=0.912; Walby, $F_{2,68}$=0.386, p=0.681; Long, $F_{2,78}$= 0.006, p=0.994; Lynne, $F_{2,67}$=0.273, p=0.762; South Rolly, $F_{2,54}$=0.156, p=0.856). Assuming that sample sizes were adequate, larger

relative telencephalon sizes of field-preserved samples do not appear to be due to the presence of adult fish.

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