Phenotypic plasticity of the threespine stickleback 
*Gasterosteus aculeatus* telencephalon in response to experience in captivity

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**Abstract** Threespine stickleback were used to examine phenotypic plasticity of telencephalon in relation to inferred ecology. Fish from derived, allopatric, freshwater populations were sampled from three shallow, structurally complex lakes with benthic-foraging stickleback (benthics) and from 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. In field-preserved and lab-held fish, the telencephalon of limnetics exhibited triangular dorsal shape, while those of benthics and sea-run fish had rounder shapes. No such pattern was detected in lab-bred fish. Within each treatment type, benthics had larger relative telencephalon sizes, using overall brain size as the covariate, than limnetics. Among field-preserved samples, sea-run fish had smaller telencephalon sizes than lake fish. Intra-population analyses of lake samples showed that field-preserved fish consistently had larger relative telencephalon sizes than lab-bred fish. The opposite was true of the sea-run population. In a separate study using one benthic population and one limnetic population, samples were preserved in the field immediately or held in the lab for 30, 60, and 90 days before they were sacrificed. In both populations, the telencephalon shapes of lab-held fish were similar to those of field-preserved fish but became progressively more like lab-bred ones over 90 days. In contrast, relative telencephalon size decreased dramatically by 30 days after which there was little change. In freshwater threespine stickleback, the telencephalon exhibits considerable phenotypic plasticity, which was probably present in the ancestor [current Zoology 58 (1): 189–210, 2012].

**Keywords** Telencephalon, Phenotypic plasticity, Brain plasticity, Spatial learning, Adaptive radiation, Hippocampus

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). The evolution of behavioral plasticity (West-Eberhard, 2003; Pigliucci and Murren, 2003; Price et al., 2003; Shaw et al., 2007) and the proximate causes of plastic changes to the brain (see Kolb et al., 2003; Curley et al., 2011; Mongiat and Schinder, 2011; Toni and Sultan, 2011) are active topics of research, but the evolution of brain plasticity remains poorly understood (see Nieuwenhuys, 1998a, 1998b; Northcutt, 2002; Striedter, 2005). There is great interest in understanding how phenotypic plasticity of the brain relates to behavioral ecology and evolution because behavior may either accelerate (Baldwin, 1896, 1902; Rau, 1933; Romer, 1958; Colbert, 1958; Weislo, 1989; West-Eberhard, 1989; Wilson and McLaughlin, 2010) or slow down (see Sulton, 1992; Schlichting, 1986) evolutionary change.

The telencephalon of threespine stickleback fish *Gasterosteus aculeatus* is 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 and Hollis, 1983), processing of sensory information (Davis and Kassel, 1983; Overmier and Hollis, 1983), mating and reproduction (Schonherr, 1955; Segarra, 1956; Demski and Beaver, 2001), social behavior (Huber et al., 1997; Kotrschal et al., 1998; Hofmann, 2001), schooling behavior (Davis and Kassel, 1983), and avoidance learning (Portavella et al., 2003, 2004; Vargas et al., 2009). The size of the telencephalon is associated
with residence in structurally complex habitats (Bauchot et al., 1977; van Staaden et 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 and Nevitt, 2003; Kihslenger et al., 2006; Burns et al., 2009; von Krogh et al., 2010; Wilson and McLaughlin, 2010), and norms of reaction vary across fishes. For example, hatchery-bred rainbow trout (Marchetti and Nevitt, 2003; Kihslenger et al., 2006) and lab-bred guppies (Burns et al., 2009) have a smaller telencephalon 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 “telencephalic plasticity”) and evolution. It is ancestrally 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. Benthic and limnetic stickleback populations represent extremes along a foraging continuum. Benthics inhabit structurally complex habitats of the littoral zone, while limnetics occur in open-water areas of lakes (Schluter and McPhail, 1992). Extreme bentic 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 al., 2001), stronger armor (Vamosi, 2002), a more streamlined body (Lavin and McPhail, 1985, 1986; Walker, 1997; Aguirre, 2009) and head (Willacker et al., 2010) form, different dental microwear (Purnell et al., 2006), and 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 prey handling (Mackney and Hughes, 1995). In addition, compared to benthics, limnetics exhibit more conspicuous courtship behavior (Foster, 1994), less male-male aggression (Scotti and Foster, 2007), and do not engage in cannibalism of young defended by conspecific males (Foster, 1994).

Fig. 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. Forty micron cross-section of telencephalon (Nissl-stained) at section S in part a (dorsal is up). The three major teost pallial subregions are the dorsomedial (Dm), dorsodorsal (Dd) and dorsolateral (Dl). The 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. From Park and Bell (2010).
Ecotypic differences in trophic structures of fishes may represent genetic divergence (McPhail, 1984, 1992; Lavin and McPhail, 1987; Hendry et al., 2002; Spoljaric and Reimchen, 2007) or phenotypic plasticity (Meyer, 1987; Wimberger, 1991; Wund et al., 2008). Ecotypic variation of telencephalon morphology is associated with foraging ecology (e.g., Bauchot et al., 1977; van Staaden et al., 1994/95; Huber et al., 1997; Pollen et al., 2007; Park and Bell, 2010; Wilson and McLaughlin, 2010), but only a handful of studies have addressed its relationship to telencephalic plasticity (e.g., Pollen et al., 2007; von Krogh, 2010; Wilson and McLaughlin, 2010; Dunlap et al., 2011; Gonda et al., 2011). Learning ability mediated by the telencephalon is important to stickleback. For example, spatial learning differences between field-caught pond and river stickleback specimens (Girvan and Braithwaite, 1998, 2000; Odling-Smee and Braithwaite, 2003a, b) were absent using lab-bred fish (Girvan and Braithwaite, 2000). Therefore, telencephalic plasticity may be substantial in this species.

The present study used threespine stickleback from Cook Inlet, Alaska to test the hypothesis that captivity affects telencephalon morphology. Park and Bell (2010) reported that the telencephala of these benthics were laterally convex and similar in relative size (standardized using brain length) to those of the limnetics, and therefore, similar findings were expected in the present work. To distinguish the importance of genetic and environmental factors on this trait, 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 before sacrifice. 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 telencephalic plasticity. These lake populations occur in a recently deglaciated region of south-central Alaska (Bell et al., 1993) that was colonized by anadromous ancestors within the last 15,000 years (Reger and Penny, 1996; Bell et al., 1993). These populations exhibit the usual ecotypic differences between benthic and limnetic populations (Walker, 1997; Purnell et al., 2006; Aguirre, 2009; Willacker et al., 2010; Park and Bell, 2010; Park, unpublished data) and have contrasting foraging behavior (S.A. Foster and J. Baker, personal communication). Therefore, these benthic and limnetic populations occur toward the opposite ends of a benthic-limnetic continuum for lake populations.

In birds and mammals, telencephalic nuclei (Buchanan et al., 2004) and the hippocampus (Mirescu et al., 2004; Day et al., 2008) of individuals raised or held in captivity typically have less neural tissue than their wild counterparts. Similarly, captive-bred fish usually have smaller telencephala than their field-preserved counterparts (Marchetti and Nevitt, 2003; Kihlslinger et al., 2006; Burns et al., 2009), and neural atrophy occurs in captive fishes (Burgess and 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. It was not immediately clear to us how telencephalon shape would be influenced by captivity, and therefore no prediction could be made about the effect of treatment on this trait.

Extant anadromous populations can be used to infer the ancestral condition of threespine stickleback traits (Bell, 1976, 1995). Field-preserved anadromous stickleback were collected from two geographically discrete sites, and parents for lab-bred fish were collected from one of these sites. Lab-held anadromous fish could not be studied because of their high mortality in captivity. The anadromous populations sampled should be similar to other anadromous populations because there is limited ecological and morphological variation among anadromous threespine stickleback populations worldwide (Bell and Foster, 1994; Walker and Bell, 2000; Colosimo et al., 2005), and the fossil record indicates that morphological traits of marine threespine stickleback have been phenotypically conservative for approximately 13 million years (Bell, 1994; Bell et al., 2009). The patterns of phenotypic plasticity expressed in ancestral, anadromous 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, telencephalic 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.

1 Materials and Methods
1.1 Sampling
Threespine stickleback were collected using 3.18 mm or 6.36 mm mesh unbaited Gee minnow traps set overnight in ≤ 1 m depth and ≤ 3 m from shore. Water temperatures were between 15° and 20°C. Stickleback samples were collected from three benthic, three limnetic,
and two anadromous populations from the Matanuska-Susitna Borough in Cook Inlet, Alaska (Table 1). 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. 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 and McPhail, 1999, 2000). 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. Relative littoral area (RLA, a measure of euphotic zone depth in a lake with lower values indicating less littoral habitat) was used to quantify habitat complexity in lakes. Park and Bell (2010) reported RLA values for lakes used in this study. Benthic populations came from high RLA lakes in which most of the bottom is within the euphotic zone and rooted vegetation is widespread; limnetics came from low RLA lakes in which most of the bottom is below this depth, limiting rooted vegetation (see Walker, 1997). Populations from these lakes exhibit the expected diet, behavior, and morphology for their eco-type (Park and Bell, 2010). 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

### Table 1  Sources and composition of threespine stickleback samples

<table>
<thead>
<tr>
<th>Population</th>
<th>Ecotype</th>
<th>Sample</th>
<th>Year</th>
<th>FM</th>
<th>n♂</th>
<th>n♀</th>
<th>Mean SL ± SE</th>
<th>Mean BS ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corcoran Lake</td>
<td>Benthic</td>
<td>FP</td>
<td>2007</td>
<td>A</td>
<td>26</td>
<td>23</td>
<td>42.353 ± 0.854</td>
<td>51.535 ± 1.062</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₃₀</td>
<td>2007</td>
<td>A</td>
<td>6</td>
<td>11</td>
<td>37.682 ± 0.413</td>
<td>47.148 ± 0.521</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₆₀</td>
<td>2007</td>
<td>A</td>
<td>6</td>
<td>9</td>
<td>40.461 ± 0.468</td>
<td>50.279 ± 0.580</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₹₀</td>
<td>2007</td>
<td>A</td>
<td>9</td>
<td>6</td>
<td>39.370 ± 0.586</td>
<td>48.786 ± 0.680</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2008</td>
<td>A</td>
<td>10</td>
<td>8</td>
<td>43.928 ± 0.937</td>
<td>53.938 ± 1.140</td>
</tr>
<tr>
<td>Mud Lake (resident)</td>
<td>Benthic</td>
<td>FP</td>
<td>2008</td>
<td>A</td>
<td>17</td>
<td>20</td>
<td>41.647 ± 1.187</td>
<td>51.464 ± 1.573</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₃₀</td>
<td>2008</td>
<td>A</td>
<td>5</td>
<td>7</td>
<td>36.886 ± 0.867</td>
<td>47.493 ± 1.075</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2009</td>
<td>A</td>
<td>9</td>
<td>6</td>
<td>39.327 ± 0.670</td>
<td>49.003 ± 0.869</td>
</tr>
<tr>
<td>Walby Lake</td>
<td>Benthic</td>
<td>FP</td>
<td>2007</td>
<td>A</td>
<td>28</td>
<td>15</td>
<td>43.568 ± 0.818</td>
<td>53.158 ± 1.027</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₃₀</td>
<td>2006</td>
<td>A</td>
<td>5</td>
<td>11</td>
<td>45.946 ± 0.710</td>
<td>56.437 ± 0.884</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2008</td>
<td>A</td>
<td>9</td>
<td>6</td>
<td>39.245 ± 0.665</td>
<td>49.605 ± 0.724</td>
</tr>
<tr>
<td>Long Lake</td>
<td>Limnetic</td>
<td>FP</td>
<td>2007</td>
<td>A</td>
<td>29</td>
<td>26</td>
<td>42.393 ± 0.872</td>
<td>51.687 ± 1.116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₃₀</td>
<td>2007</td>
<td>A</td>
<td>5</td>
<td>10</td>
<td>41.333 ± 0.553</td>
<td>51.418 ± 0.689</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₆₀</td>
<td>2007</td>
<td>A</td>
<td>11</td>
<td>4</td>
<td>40.378 ± 0.894</td>
<td>50.216 ± 1.186</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₉₀</td>
<td>2007</td>
<td>A</td>
<td>7</td>
<td>8</td>
<td>40.065 ± 0.886</td>
<td>49.308 ± 1.143</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2008</td>
<td>A</td>
<td>5</td>
<td>9</td>
<td>45.189 ± 1.344</td>
<td>56.245 ± 1.738</td>
</tr>
<tr>
<td>Lynne Lake</td>
<td>Limnetic</td>
<td>FP</td>
<td>2007</td>
<td>A</td>
<td>32</td>
<td>10</td>
<td>45.259 ± 0.723</td>
<td>54.116 ± 0.916</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₃₀</td>
<td>2006</td>
<td>A</td>
<td>10</td>
<td>6</td>
<td>36.840 ± 1.210</td>
<td>43.996 ± 1.427</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2008</td>
<td>A</td>
<td>9</td>
<td>6</td>
<td>41.863 ± 1.254</td>
<td>51.843 ± 1.649</td>
</tr>
<tr>
<td>South Rolly Lake</td>
<td>Limnetic</td>
<td>FP</td>
<td>2008</td>
<td>A</td>
<td>22</td>
<td>10</td>
<td>47.370 ± 1.102</td>
<td>58.358 ± 1.308</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LH₃₀</td>
<td>2008</td>
<td>A</td>
<td>12</td>
<td>6</td>
<td>40.987 ± 0.860</td>
<td>51.590 ± 0.985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2009</td>
<td>A</td>
<td>5</td>
<td>5</td>
<td>43.733 ± 0.666</td>
<td>54.146 ± 0.906</td>
</tr>
<tr>
<td>Rabbit Slough</td>
<td>Anadromous</td>
<td>FP*</td>
<td>2003</td>
<td>B</td>
<td>15</td>
<td>14</td>
<td>68.256 ± 0.663</td>
<td>84.822 ± 0.978</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FP*</td>
<td>2007</td>
<td>A</td>
<td>15</td>
<td>0</td>
<td>67.511 ± 0.431</td>
<td>83.066 ± 0.536</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB</td>
<td>2007</td>
<td>A</td>
<td>15</td>
<td>12</td>
<td>46.613 ± 0.620</td>
<td>57.057 ± 0.779</td>
</tr>
<tr>
<td>Mud Lake</td>
<td>Anadromous</td>
<td>FP</td>
<td>2003</td>
<td>B</td>
<td>14</td>
<td>15</td>
<td>68.877 ± 0.564</td>
<td>86.293 ± 0.786</td>
</tr>
</tbody>
</table>

Samples include: FP, field-preserved; LH₃₀, 30-day lab-held; LH₆₀, 60-day lab-held; LH₉₀, 90-day lab-held, and LB, lab-bred. Symbols are as follows: Year, collection year; FM, fixation method (see Materials and Methods); n, sample size by sex; SL, standard length; BS, body centroid size, and SE, standard error. * Samples that were pooled.
population, but the two are reproductively isolated (Karve et al., 2008; Bell et al., 2010), and the anadromous Mud Lake 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 and the University of Alaska Anchorage.

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 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 indistinguishable statistically (see Lisney and Collin, 2006; Yopak et al., 2007). We validated these results for stickleback by comparing anadromous males from Rabbit Slough that were preserved in 3.7% formaldehyde (n=15) or 4% paraformaldehyde (n=15), and a common Principal Components Analysis (see Results below) did not detect significant differences for telencephalon shapes along PC1 (Discriminant Function Analysis: $\chi^2=0.414$, $P=0.520$, $df=1$). Similarly, relative telencephalon size did not differ between samples using either fixative (ANCOVA: $F_{1,27}=0.441$, $P=0.512$). Thus, samples using both fixatives 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, 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 seawater, which was prepared using Instant Ocean® sea salt. Each aquarium was 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 aquaria were structurally bare and did not include anything other than the filtration equipment. Fish were fed daily with thawed, frozen adult brine shrimp by depositing the food onto the water surface. Generally, there were 25-30 fish per aquaria. All aquarium conditions were standardized across populations and treatments. 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 of lab-held fish from Corcoran (benthic) and Long (limnetic) lakes were also sacrificed at 30 and 60 days. The fish that were sacrificed came from all the aquaria in which they were held, and therefore, each sample came from fish held under the same set of conditions. All lab-held fish were sacrificed by overdose in MS-222 and preserved in 4% paraformaldehyde in 0.1 M PBS.

Lab-bred lacustrine fish came from the six lakes, and lab-bred anadromous fish came from Rabbit Slough. 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. Artificial mass crosses were performed at the University of Alaska Anchorage. For each population, ten sexually mature fish of each sex were anesthetized in MS-222. Sperm were obtained by cutting open the abdomen of ten males and placing their 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 their eggs into a few drops of 3 ppt artificial seawater 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 seawater over them and removing the fluid, testis debris, and ovarian mucus. They were kept in plastic culture dishes with 3 ppt artificial seawater in a constant-temperature incubator at 19°C. Within three days after fertilization, the eggs were
transferred to 35 or 50 ml vials nearly filled with 3 ppt artificial seawater at 19°C, chilled to 5°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 randomly selected embryos were transferred into each of five plastic culture dishes with 3 ppt seawater and placed into an incubator at 19°C. Water was changed daily. Three to five days after the fry hatched and their yolk sacs had been 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. The aquaria were structurally identical to those used to house lab-held fish. Generally, there were 25–30 fish per aquarium. Aquarium conditions were standardized across populations and treatments. 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, which were deposited on the water surface. At fourteen months post-hatching, all lab-bred specimens were sacrificed by overdose in MS-222 and preserved in 4% paraformaldehyde in 0.1 M PBS.

1.2 Brain imaging and geometric morphometrics

The methods to digitize and place landmarks on the dorsal aspect of the telencephalon were explained in Park and Bell (2010). Briefly, brains 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 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 with a 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.

Variation of the left lobe of the telencephalon was quantified using the semi-landmark technique of geometric morphometrics (Rohlf and Marcus, 1993; Zelditch et al., 2004). This technique allows superimposition of shapes with few discrete landmarks but requires fixed terminal landmarks (i.e., LM 1 and LM 14; Bookstein, 1996/97). Each intermediate semi-landmark can “slide” parallel to a line connecting the two adjacent fixed or sliding semi-landmarks. Using tpsDig version 2.05 (Rohlf, 2006), a pair of fixed extreme landmarks (i.e., LM 1, LM 14) was digitized, 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 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; and 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. TpsRegr 1.31 (Rohlf, 2005a) was used to obtain 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.

1.3 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 with a low-dimensional Euclidean space representing as much of the variation as possible. All specimens (Table 1) 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 rela-
tive 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 compared to 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 ecotypic and treatment differences. Specific comparisons are given in the Results.

### 1.4 The standardizing variable

Brain substructures are typically size-standardized using a measure of body size or overall brain size (see Striedter, 2005). Overall brain size should be a more reliable covariate than body size because brain structures are more anatomically and functionally integrated with each other than with body size (see Broglio et al., 2003; Rodriguez et al., 2005), and absolute brain and body sizes have become decoupled in many vertebrate groups (see Striedter, 2005). In addition, regression of telencephalon size on body size was not statistically significant for some samples in the present analysis, and therefore, body size could not be used as a size standardizing variable in some cases. However, regression of telencephalon size on overall brain dorsal area was significant for all samples (see Online Supplement) and thus was used as a size standard to calculate relative telencephalon size in all analyses.

Overall brain size was estimated using the dorsal area of the brain. 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 dorsal width of the brain. Thus, the dorsal area of the brain that is intersected by this length, including 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 populations (see Online Supplement).

### 1.5 Relative telencephalon size - univariate and bivariate statistical analyses

Univariate and bivariate statistical analyses were performed on neuroanatomical traits. All telencephalon measurements were natural log-transformed and size standardized. Statistical analyses were conducted using Biostat version 3.30. 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 among treatments. 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. However, the regression slopes 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 mm²). A t-test was used to compare predicted means.

## 2 Results

### 2.1 Telencephalon shape - PCA using all samples

A single PCA of telencephalon shapes was performed using all samples (Table 1), and all shape analyses were based on values from this PCA. 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 out-
line is more concave, triangular, and elongate along the dorsal midline. At the negative end of PC1, the outline of the telencephalon is more 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). None the remaining 22 PC axes accounted for more than 12.6% of additional variation and were ignored in subsequent analyses.

Discriminant Function Analysis (DFA) was used to analyze telencephalon shapes of fish from field-preserved, lab-held (90-day), and lab-bred samples. Statistical details are given in Table 2. Telencephala of field-preserved fish occupied a single quadrant exclusively (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. DFA indicated that field-preserved, lab-held, and lab-bred samples all differed significantly along both PC1 and PC2 from each other.

A DFA detected a difference in telencephalon shapes between benthics and limnetics along PC1 for field-preserved and for lab-held samples. The general pattern within both treatment groups was similar; benthics have rounder shapes than limnetics for both treatments. However, no ecotypic pattern was detected for lab-bred samples. PC2 did not distinguish ecotypes in any treatment and therefore was not used 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 relatively convex and laterally extended, which was similar but not identical to those of field-preserved benthic populations. Among PC1, telencephalon shapes of field-preserved anadromous fish from Rabbit Slough and Mud Lake differed significantly from those of field-preserved, lake-fish samples (Table 2).

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 and of lab-bred lake fish along PC1.

### 2.2 Relative telencephalon size - field-preserved, lab-held (90 days), and lab-bred samples

ANCOVA also indicated significant differences between ecotypes and among treatments within ecotypes. Relative telencephalon sizes were greater in pooled benthics than in pooled limnetics in the field-preserved ($F_{1,259}=8.426, P<0.01$), lab-held ($F_{1,95}=16.599, P<0.001$), 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 separately. An ANCOVA detected differences in relative telencephalon sizes among lake samples within each treatment (field-preserved, $F_2, 251=16.565, P<0.001$; 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 infer which population means differed from others (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-preserved 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 both Rabbit Slough ($F_{1,299}=81.249, P<0.001$) and Mud

### Table 2  Discriminant function analysis of telencephalon shape differences between ecotypes, treatments, and other samples

<table>
<thead>
<tr>
<th>Comparison</th>
<th>DFA (PC1)</th>
<th>DFA (PC2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP&lt;sub&gt;Lake&lt;/sub&gt; x LH&lt;sub&gt;Lake&lt;/sub&gt;</td>
<td>$\chi^2=130.679, P&lt;0.001$</td>
<td>$\chi^2=55.447, P&lt;0.001$</td>
</tr>
<tr>
<td>FP&lt;sub&gt;Lake&lt;/sub&gt; x LB&lt;sub&gt;Lake&lt;/sub&gt;</td>
<td>$\chi^2=68.747, P&lt;0.001$</td>
<td>$\chi^2=99.207, P&lt;0.001$</td>
</tr>
<tr>
<td>LH&lt;sub&gt;Lake&lt;/sub&gt; x LB&lt;sub&gt;Lake&lt;/sub&gt;</td>
<td>$\chi^2=11.212, P&lt;0.001$</td>
<td>$\chi^2=13.934, P&lt;0.001$</td>
</tr>
<tr>
<td>FP&lt;sub&gt;Ben&lt;/sub&gt; x FP&lt;sub&gt;Limn&lt;/sub&gt;</td>
<td>$\chi^2=13.253, P&lt;0.001$</td>
<td>$\chi^2=0.822, P=0.365$</td>
</tr>
<tr>
<td>LH&lt;sub&gt;Ben&lt;/sub&gt; x LH&lt;sub&gt;Limn&lt;/sub&gt;</td>
<td>$\chi^2=3.883, P=0.05$</td>
<td>$\chi^2=0.0249, P=0.875$</td>
</tr>
<tr>
<td>LB&lt;sub&gt;Ben&lt;/sub&gt; x LB&lt;sub&gt;Limn&lt;/sub&gt;</td>
<td>$\chi^2=1.057, P=0.304$</td>
<td>$\chi^2=3.02, P=0.0824$</td>
</tr>
<tr>
<td>FP&lt;sub&gt;Anad(RS)&lt;/sub&gt; x FP&lt;sub&gt;Lake&lt;/sub&gt;</td>
<td>$\chi^2=59.690, P&lt;0.001$</td>
<td>N/A</td>
</tr>
<tr>
<td>FP&lt;sub&gt;Anad(Mud)&lt;/sub&gt; x FP&lt;sub&gt;Lake&lt;/sub&gt;</td>
<td>$\chi^2=84.884, P&lt;0.001$</td>
<td>N/A</td>
</tr>
<tr>
<td>FP&lt;sub&gt;Anad(RS)&lt;/sub&gt; x LB&lt;sub&gt;Anad(RS)&lt;/sub&gt;</td>
<td>$\chi^2=69.464, P&lt;0.001$</td>
<td>N/A</td>
</tr>
<tr>
<td>LB&lt;sub&gt;Anad(RS)&lt;/sub&gt; x LB&lt;sub&gt;Lake&lt;/sub&gt;</td>
<td>$\chi^2=66.835, P&lt;0.001$</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Abbreviations: FP, field-preserved; LH, lab-held (90-day), and LB, lab-bred. Subscripts are: Lake, all lake samples (pooled); Ben, all benthic lake samples (pooled); Anad(RS), sea-run fish from Rabbit Slough, and Anad(Mud), sea-run fish from Mud Lake. df = 1 for each comparison. N/A, not applicable because PC2 did not explain ecotypic variation and therefore these analyses were not pertinent to main findings.
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, 90-day lab-held, or lab-bred treatments. The symbols for ecotypes are in the inset legend of the upper plot. Lake sites: 1, Long; 2, Lynne; 3, South Rolly; 4, Corcoran; 5, Mud; 6, Walby.

Intra-population treatment effects were studied separately for each population (Fig. 5). An ANCOVA detected differences in relative telencephalon sizes among field-preserved, 90-day lab-held, or lab-bred treatments. The symbols for ecotypes are in the inset legend of the upper plot. Lake sites: 1, Long; 2, Lynne; 3, South Rolly; 4, Corcoran; 5, Mud; 6, Walby.

Relative telencephalon sizes of field-preserved and lab-bred anadromous fish from Rabbit Slough were compared. 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 (see Park and Bell, 2010). Linear regression equations were statistically significant for field-preserved (\(F_{1,42}=176.193, P < 0.001\)) and lab-bred (\(F_{1,25}=47.321, P < 0.001\)) anadromous samples. For each sample, a predicted telencephalon size was calculated using the collective mean brain area based on both anadromous samples (\(BA=17.841 \text{ mm}^2\)). Contrary to expectations, lab-bred anadromous fish had larger relative telencephalon sizes than those of their field-preserved counterparts (\(t_{69}=7.399, P < 0.001\)).

### 2.3 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 telencephalon 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: \(\chi^2=10.880, P < 0.001, df=1\); 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: $\chi^2 = 0.0292$, $P = 0.86$, $df = 1$) or Long (DFA: $\chi^2 = 0.0127$, $P = 0.91$, $df = 1$) 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 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 significantly 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 significantly smaller (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 the Corcoran and Long lake populations, the telencephala of wild fish appeared to undergo a drastic decrease in relative size after 30 days of captivity, which was not followed by substantial additional reduction for up to 90 days, and they resembled those of lab-bred counterparts.

**Fig. 5** Comparison of relative telencephalon size among samples from wild and captive samples in three experimental treatments

Ninety-five percent comparison limits for size-adjusted, natural log-transformed telencephalon centroid size using the GT2-method test for unplanned comparisons. The name of each lake site is in its corresponding plot. Samples: FP, field-preserved; LH$_{90}$, 90-day lab-held, and LB, lab-bred.
Fig. 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 (LH₃₀), 60 (LH₆₀), and 90 days (LH₉₀). Deformation grids describe the shapes at the ends of each axis and the corner of negative PC1 and PC2 in the plot. The mean shape of each field-preserved sample is given in the plot. The symbols for the different treatments are in the inset legend. Plots (a) and (b) are a common morphospace.
3 Discussion

We investigated telencephalic phenotypic plasticity in field-preserved, lab-held, and lab-bred benthic and limnetic lake stickleback and in anadromous stickleback. As before (Park and Bell, 2010), the telencephalons of field-preserved benthics were convex compared to the more concave and triangular shapes of limnetics, but these ecotypic shape differences disappeared in lab-bred fish. Benthics also had larger relative telencephala than limnetics across all treatments, and this difference was most pronounced among lab-bred samples. Among samples from each lake, field-preserved fish showed a strong trend toward larger telencephalon sizes than lab-held and lab-bred fish. Taken together, these results indicate that the size and shape of the telencephalon is generally phenotypically plastic in the threespine stickleback, but there is also a genetic component, as benthics have a larger telencephalon than other ecotypes, regardless of experience.

3.1 Potential confounding variables

Potential confounding variables that could explain findings in the present work are sexual dimorphism, selective mortality, inclusion of sexually mature fish in field-preserved samples, food quality, and capture method. In stickleback, males tend to have more triangular telencephalon shapes and larger relative telencephala than females (Park and Bell, 2010). The sex ratios in our samples were roughly similar (Table 1), so sexual dimorphism is unlikely to explain our general findings.

Ecotypic differences in the telencephalon shapes of field-caught fish could have been due selective mortality of specimens with inappropriate telencephalon phenotypes prior to capture, which would not occur in lab-bred fish (see Brown and Braithwaite, 2004). Determining the effect of selective mortality would be difficult in any comparison field-preserved and lab-bred fish, but other studies of brain plasticity did not address this issue either (Marchetti and Nevitt, 2003; Kihshlinger et al., 2006). However, if phenotypic plasticity was not important in stickleback, the telencephalons of field-preserved and lab-held fish, both of which were field-caught, should have been similar, but they had different shapes (Fig. 3) and relative sizes (Fig. 5). While our methods cannot exclude the possibility that selective mortality in natural populations caused differences between field-preserved and lab-bred fish, those between field-preserved and lab-held fish indicate that telencephalic plasticity occurs in stickleback.

Sexual maturation can trigger changes in brain morphology (e.g., Jacobs et al., 1990; Jacobs and Spencer, 1994; Clayton et al., 1997; Sherry, 1998). Field-preserved fish in our study had different telencephalon shapes (Fig. 3) and larger relative sizes than lab-held and lab-bred fish (Fig. 5). These differences could be explained by inclusion of adult fish in field-preserved samples. Threespine stickleback from Cook Inlet lakes typically mature at two years old, during their third summer of life (Havens et al., 1984; Heins et al., 1999; Baker et al., 2008), but our lab-held and lab-bred fish were sacrificed during their second summer without entering reproductive condition. In contrast, field-preserved samples included a broad range of body sizes to account for potential variability due to limited sample sizes in lab-held and lab-bred samples. Field-preserved samples undoubtedly included one year-old juveniles and two year-old adults. The larger relative telencephalon of field-preserved fish could reflect inclusion of adults in the field-preserved sample. However, a preliminary analysis for homogeneity of slopes failed to detect differences in the telencephalon growth trajectories using brain area as the covariate in field-preserved, lab-bred, and lab-held fish for each population (see Online Supplement), 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, but future work will need to include more rigorous tests to fully address this concern.

The two remaining factors could explain the larger relative telencephalon sizes of field-preserved fish compared to those of lab-held and lab-bred ones. Field-preserved fish probably consumed a more nutrient-rich diet than their lab counterparts, which could have resulted in their larger telencephalon sizes. It is also possible that minnow traps tend to capture bolder or more exploratory fish (see Biro and Dingemanse, 2009; Budaev, 1997) that are better spatial learners and have larger telencephala than shy individuals (see Burns and Rodd, 2008). Due to practical limitations, either factor could not be rigorously addressed in the present work. Future work will need to address the potential impact of differing diets and sampling methods on telencephalon morphology.

3.2 Telencephalon shape in relation to inferred ecology

The dorsal shapes of the telencephala in field-preserved benthic stickback are generally round while those of limnetics are more triangular, confirming previous results (Park and Bell, 2010). Ecotypic differences in telencephalon shape may reflect differences in relative size of telencephalic subregions that mediate ecologically-important behaviors. For example, the greater telencephalon convexity of field-preserved benthics compared to limnetics (Fig. 3) may indicate greater volume of the dorsolateral region (Dl) of the telencephalon (Park and Bell, 2010), which processes spatial learning in fishes (Salas et al., 1996a, 1996b; Vargas et al., 2000, 2009; Rodriguez et al., 2002; Broglio et al., 2003; Northcutt, 2006). Spatial learning typically varies with ecology in vertebrates (Sherry, 1998) and depends considerably on phenotypic plasticity (Juraska et al., 1985, 1989; Vargas et al., 2000). Limnetic stickback exhibit poor spatial learning compared to benthics (Odling-Smeek and Braithwaite, 2003a; Odling-Smeek et al., 2008; Park, 2011). Dl is homologous to the tetrapod hippocampus (Vargas et al., 2000, 2009). In birds and mammals, greater hippocampal size is associated with superior spatial learning (O’Keefe and 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 and Krebs, 1994; Healy, 1996, 1998; Krebs and Davies, 1997; Healy et al., 2005). Similarly, male kangaroo rats Dipodomys and meadow voles Microtus have larger home-ranges, better spatial learning ability, and a larger hippocampus than sedentary females (Jacs and Spencer, 1994; Jacobs et al., 1990). Like the hippocampus, larger Dl size in the telencephalon is associated with larger home-range sizes in bennies (Caniero et al., 2001) and residence in structurally complex habitat in cichlids (Shumway, 2008). In stickback, field-caught bennies which occupy more complex littoral environments have rounder telencephalon shapes (Park and Bell, 2010) and superior spatial learning ability than field-caught limnetic (Odling-Smeek et al., 2008; Park, 2011) and river (Girvan and Braithwaite, 1998) stickback (see Park and Bell, 2010). Therefore, wild bentic stickback may have a larger Dl, accounting for better spatial learning than in limnetics.

Other behavioral traits may be associated with telencephalon shape differences between bennies and limnetics. Compared to field-caught bennies, field-caught limnetics also exhibit shorter memory for handling prey (Mackney and Hughes, 1995), less male-male aggression (Scotti and Foster, 2007), and more conspicuous courtship behavior (Foster, 1994). Differences in aggression among stickback populations are heritable (Bell, 2005), but the effect of these behavioral differences on 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.

Going from field-preserved to lab-held and lab-bred samples, there was a progressive shift towards more negative values along both PC axes for telencephalon shape (Fig. 3), indicating phenotypic plasticity. Although further study of reaction norms of telencephalon shape would be desirable, we believe that phenotypic plasticity of telencephalon shape in threespine stickback may well be great enough to completely mask heritable differences among ecotypes. Thus, experience may be an important cause of numerous observed ecotypic behavioral differences among threespine stickback populations.
3.3 Telencephalon size in relation to inferred ecology

Park and Bell (2010) did not detect a difference in relative telencephalon size between field-preserved benthics and limnetics using brain length as the covariate. The present study included a subset of their populations, substituted dorsal brain area as the covariate to standardize telencephalon centroid size, and found that field-caught benthics had larger relative telencephalon sizes. The field-caught sample from Lynne Lake (limnetic) had a greater mean relative telencephalon size than those from two bentic populations. The greater ratio of males to females in this sample may explain these results because males tend to have larger relative telencephalon size (Park and Bell, 2011). It is also possible that the Lynne Lake population is an outlier compared to other limnetic populations. While the RLA value for Lynne Lake was not unlike those of other deep lakes, 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. However, the exceptional Lynne Lake population could indicate that there is high variability among lakes even within forms, and analysis of different sets of bentic (e.g., Tern Lake, Willow Lake) and limnetic (e.g., Matanuska Lake, Stormy Lake) populations by the present study and Park and Bell (2010) caused the difference in results between the studies.

There may also be more substantive causes for differences between studies. While brain area and brain length should be highly correlated, there may be ecotypic differences along the width of the brain that are not captured by length. Compared to fish species from structurally complex habitats, pelagic fishes tend to have larger eyes and larger optic lobes (Kotrschal et al., 1998). Limnetic stickleback occupy open waters and also have larger eyes than benthics (reviewed in Bell and Foster, 1994; McPhail, 1994; Walker, 1997); therefore, limnetics may also have larger optic lobes. Thus, relatively larger optic lobes in limnetics could increase their dorsal brain area and account for contrasting findings of 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 from open-water habitats, those of structurally complex habitats have greater relative telencephalon sizes (Bauchot et al., 1977; van Staaden et al., 1994,1995; Huber et al., 1997), which may cause 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 for spatial learning, parental care, or social behavior.

In contrast to field-caught fish, the largest relative telencephalon sizes among lab-bred fish occur exclusively in benthics. This difference can be explained by reduction of the relative rank of the Lynne Lake sample by two ranks (Fig. 4). Because lab-bred fish were raised under nearly identical conditions (See Materials and Methods), differences between relative telencephalon size of bentic and limnetic stickleback may be heritable. Nonetheless, the general pattern of population differences differ between field-preserved and lab-bred fish. Thus, phenotypic plasticity may strongly impact 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 application of such results to variation among natural populations may be obscured by phenotypic plasticity.

Field-preserved fish consistently had larger relative telencephalon sizes than fish from the same population held in the lab for 90 days or bred in the lab. These differences could be due to greater sensory stimulation or accelerated growth of brain substructures for other reasons (see Kihslinger et al., 2006). Environmental enrichment (Juraska et al., 1985, 1989; van Praag et al., 2000; Rampon et al., 2000; Faherty et al., 2003) and maternal contact early in life (Mirescu et al., 2004) induce cell proliferation and dendritic growth in the hippocampus of mammals. Similarly, experience with food storage and retrieval (Patel et al., 1997) and successful nest searching (Day et al., 2008) stimulates brain development in birds. In fishes, experience with spatial tasks induces protein synthesis in the dorsolateral region of the telencephalon (Vargas et al., 2000). Thus, enhanced spatial learning could increase relative telencephalon size of field preserved stickleback.

However, several differences between field and laboratory conditions might influence relative telencephalon size by either reducing its growth or increasing growth of other parts of the brain. For example, stress from starvation or corticosterone administration decreased the size of brain nuclei in adult birds (Bucanahan et al., 2004). Stress from elevated temperature caused...
reduction of neuronal cell number in pejerrey fish (*Odontesthes bonariensis*; Miranda et al., 2003). Greater exposure to aggression reduced neuron size in pupfish (*Cyprinodon nevadensis*; Lema, 2006). In jewel fish *Hemicromis bimaculatus*, crowding induced loss of dendritic spines (Burgess and Coss, 1982), and in nine-spine stickleback *Pungitius pungitius*, pond fish reared in a group had smaller overall brain sizes than those raised individually (Gonda et al., 2009). Thus, smaller relative telencephalon size of lab-bred and lab-held stickleback in the current study may reflect environmental differences other than opportunities for spatial learning.

### 3.4 The effect of time in captivity on telencepha-lon 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 fish held in aquaria for 30, 60, and 90 days. After 30 days, the lab-held fish had dramatically smaller relative telencephalon size, with minimal reduction after 60 and 90 days. In contrast, telencephalon shapes changed gradually throughout this period, with telencephala of lab-held fish becoming progressively more elongate and laterally rounder anteriorly (i.e., landmarks 8-12), eventually resembling that of lab-bred fish. Given that relative telencephalon sizes were largest in field-preserved fish, rounder anterior telencephalon features of 90-day lab-held fish could be due to the loss of neural tissue in posterior areas. However, this shape change cannot be due only to tissue loss because the shape continued to change after the size became stable. Thus, the reasons for change in telencephalon shape of lab-held fish remain unclear. However, the contrast between abrupt change of telencephalon size and gradual change of shape in lab-held fish suggests that some parts of the telencephalon responded sooner than others during captivity. 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 telencephalic tissue occurs gradually or during critical periods (see Thorpe, 1958; Penfield and Roberts, 1959; Almlí and Finger, 1987).

### 3.5 The ancestral condition for phenotypic plasticity of the telencephalon

Anadromous stickleback can be used to infer the ancestral condition for traits of derived freshwater populations (Bell, 1976, 1995). They feed on plankton at sea (Mackney and Hughes, 1995), and their trophic morphology resembles that of limnetics (McPhail, 1994). There is relatively little information on the biology of threespine stickleback at sea, but they have often been reported taking large samples in deep waters hundreds of kilometers offshore in the Gulf of Alaska (Mackney and Hughes, 1995). Several authors have reported taking large samples in deep waters hundreds of kilometers offshore in the Gulf of Alaska (Mecklenburg et al., 2002). Cowen et al. (1991) showed that juvenile stickleback may move off shore extremely rapidly, suggesting that it is an active process. Thus, while it would be impossible to state that all anadromous *G. aculeatus* live in the open ocean, there is ample evidence that movement into deep, offshore habitats is deliberate and common. Generally, threespine stickleback from Cook Inlet reproduce after two years (Havens et al., 1984; Heins et al., 1999; Baker et al., 2008), 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 extreme negative values for telencephalon shape on PC1 (Fig. 3), which corresponds to very convex shapes resembling those of benthics more so than limnetics. This trait may be the ancestral condition that was retained in benthic populations (see Park and Bell, 2010). Curiously, telencephala of lab-bred anadromous fish 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, (see also Park and Bell, 2010). Contrary to expectations based on comparisons of field-preserved and lab-bred specimens, lab-bred anadromous fish had larger relative telencephalon sizes than field-preserved counterparts and lab-bred lake fish. While these results are paradoxical, they are consistent with findings from ninespine stickleback (*Pungitius pungitius*; Gonda et al., 2009). One possible explanation for these results 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. Anadromous salmon undergo immense muscular and physiological degradation during migration 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...
3.6 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 adaptive (Swain, 1992; also see Gonda et al., 2011). Environmental effects may be particularly important for the telencephalon. Field-preserved stickleback consistently had larger relative telencephalon sizes than lab-held or lab-bred fish, and ecotypic telencephalon shape differences were absent in lab-bred fish. These findings are consistent with studies using hatchery-bred rainbow trout (Marchetti and Nevitt, 2003; Kihslinger et al., 2006) and lab-bred guppies (Burns et al., 2009) and with reports that domesticated fishes (Mayer et al., 2011), birds, and mammals (Ebinge and Rohrs, 1995; Kruska, 1988) generally have smaller relative brain sizes than their wild counterparts.

Phenotypic plasticity of the telencephalon may be more important for benthic stickleback than for limnics, because they probably utilize spatial learning to a greater extent (Park, 2011; see also Odling-Smee et al., 2008). In support of this hypothesis, captive cowbirds had smaller relative hippocampal sizes than field-caught counterparts presumably due to limited spatial learning (Day et al., 2008). The importance of telencephalic 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, telencephalic plasticity should be substantial in the anadromous ancestor of freshwater stickleback.

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 and 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; Wilson and McLaughlin, 2010). For example, behavioral plasticity in mating behaviors (e.g., zigzag dance) in the threespine stickleback occurs in ancestral populations and may have influenced evolution of heritable differences among derived freshwater populations (Shaw et al., 2007). Similarly, changes in telencephalon morphology may occur in these populations after demands for spatial learning have been imposed. However, if telencephalon morphology changes first, it would inevitably be followed by a change in affected behaviors (Romer, 1958; Colbert, 1958). For example, an unfavorable environmental impact on fish neurology, as often occurs during extended captivity (see Burgess and Coss, 1982; Miranda et al., 2003; Mirescu et al., 2004; Buchanan et al., 2004), may influence 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 and Nevitt, 2003; Kihslinger et al., 2006; Burns et al., 2009) buffers against evolutionary change (see Sulton, 1992; Schlichting, 1986). Thus, many critical questions about the relationship between brain plasticity and evolution of the brain and behavior remain unanswered. The threespine stickleback provides a powerful model system with which to seek answers.

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References


Colosimo PF, Hosemann KE, Balabhadra S, Villarreal G Jr, Dickson M et al., 2005. Widespread parallel evolution in


Odling-Smee LC, Braithwaite V A, 2003b. The influence of habitat


Spoljaric MA, Reimchen TE, 2007. 10,000 years later: Evolution of body shape in Haida Gwaii three-spined stickleback. J. Fish


