Tinkering with the axial skeleton: vertebral number variation in ecologically divergent threespine stickleback populations

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Understanding how the vertebral column is impacted as populations adapt to different habitats favouring distinct body forms can provide insight into the origin of evolutionary diversity in the axial skeleton. We examined variation of vertebral number and body shape in morphologically and ecologically divergent anadromous, benthic, and limnetic threespine stickleback populations from Alaska. Variation in vertebral number was substantial and was associated with body shape variation. Both vertebral homeosis and region-specific changes in vertebral number were present. Sexual dimorphism in vertebral number was significant and resulted from vertebral homeosis; females had more abdominal vertebrae and males more caudal vertebrae, although total vertebrae did not differ. Body elongation was associated with an increase in vertebral number, especially in the caudal region, although this varied among populations. Benthics tended to have less caudal vertebrae than limnetics and anadromous stickleback generally had more abdominal vertebrae than either, although this also varied among populations. Variation among individuals indicated that external similarity in body form masks significant vertebral variation in stickleback. Although more research on the underlying mechanisms and functional significance is needed, our findings highlight the potential of the threespine stickleback as a model for studying the evolution of the vertebrate axial skeleton. © 2014 The Linnean Society of London, Biological Journal of the Linnean Society, 2014, 113, 204–219.


INTRODUCTION

Understanding how the diversity of animal form has arisen is a major goal of evolutionary biology. Fishes constitute the most diverse group of vertebrates, with over 28,000 described species and counting (Nelson, 2006). With this taxonomic diversity comes a great diversity of body forms. How the axial skeleton evolves in response to selection for different body shapes varies among fish groups. Ward & Brainerd (2007) analyzed vertebral number variation in elongate fishes representing highly divergent taxonomic lineages and found that fish bodies can increase in length by increasing the number of vertebrae, increasing the length of vertebrae, or through a combination of both. There is also regional specificity, such that the number of abdominal and caudal vertebrae can vary independently in different lineages. Recent advances in Evo-Devo are making the study of the molecular mechanisms responsible for changes in body form and axial patterning tractable. Several recent studies present candidate genes for the evolution of vertebral number in fishes (Ward & Mehta, 2010; Kimura, Shinya & Naruse, 2012; Berner et al., 2014). There have also been many recent studies that have focused on the molecular mechanisms through which the number of body segments is regulated and the identity of body segments is established in vertebrates (Gomez et al., 2008; Gomez & Pourqué, 2009; Mallo, Wellik & Deschamps, 2010). Thus, there has been significant progress in understanding both major patterns of divergence in axial skeleton evolution among fishes and in understanding the specific
genes and developmental pathways regulating the number and identity of body segments.

A notable gap in current knowledge of the evolution of fish body form is that related to microevolutionary change in the axial skeleton, especially in association with adaptation to different habitats. Research in this area could provide insight into the mechanisms at play during the earliest stages of evolutionary divergence, when allele frequencies are changing and incipient species begin their journeys down different evolutionary trajectories. The threespine stickleback, *Gasterosteus aculeatus*, is a promising system in which to study of the earliest stages of evolution of the axial skeleton given the morphological and ecological diversity documented (Bell & Foster, 1994; Östlund-Nilsson, Mayer & Huntingford 2006; Hendry et al., 2009) and the recent development of genomic tools (Kingsley et al., 2004; Hohenlohe et al., 2010; Jones et al., 2012). Stickleback are primitively oceanic, entering freshwater only to reproduce. As glaciers retreated over much of the northern hemisphere beginning approximately 20 000 years ago, oceanic stickleback colonized newly-formed lakes and streams and established resident populations that adapted to their novel surroundings. The differences among populations are numerous, although one of the most obvious is divergence in body shape. Body shape divergence in response to adaptation to benthic (close to shore, shallow water) and limnetic (deep, open water) lake habitats has been particularly well studied, has evolved repeatedly in different parts of the world (McPhail, 1984; Walker, 1997; Aguirre, 2009), and is known to have a genetic basis (Spoljaric & Reimchen, 2007; Albert et al., 2008). At one ecological extreme, fishes that adapt to shallow environments evolve deeper bodies that allow greater maneuverability in structurally complex habitats and are called ‘benthics’. At the other extreme, fish that adapt to deep water environments spend most of their time in the water column and evolve more elongate bodies that reduce drag facilitating prolonged swimming. These fish are called ‘limnetics’. How this evolution of body shape and adaptation to different ecological niches impacts upon the axial skeleton is unclear, although previous studies provide some insight.

Surveys of variation in vertebral number in stickleback populations indicate that there is substantial phenotypic variation for this trait in nature. Environmental factors such as temperature can influence stickleback vertebral phenotypes as they do in other fishes (Jordan, 1891; Lindsey, 1962); however, variation in the axial skeleton of stickleback populations appears to have a moderate to strong genetic basis (Ahn & Gibson, 1999a; Hermida et al., 2002; Alho, Leinonen & Merilä, 2011; Berner et al., 2014). Evidence that natural selection impacts upon vertebral number variation among stickleback populations in nature has been documented (Reimchen & Nelson, 1987; Swain, 1992a, b). Correlations between vertebral number and ecology have been found, with elongate limnetic stickleback tending to have more vertebrae (Reimchen & Nelson, 1987; Ahn, 1998), although this does not appear to be universally true (Ahn, 1998). As is the case with many morphological traits in stickleback, sexual dimorphism in vertebral number has also been documented (Lindsey, 1962; Reimchen & Nelson, 1987).

In the present study, we build on previous research and examine variation and covariation of vertebral number and body shape in multiple, well-characterized populations of anadromous, benthic, and limnetic stickleback from Alaska. This research expands beyond previous efforts by providing the first detailed examination of body region (abdominal and caudal) specific variation in vertebral number among replicate populations belonging to three different ecormorphs, taking sex into account. Previous efforts have focused on total vertebral number, have not taken sex into account, and/or involved only a single population. By including all these factors in a single study, their relative importance on variation in vertebral phenotypes in wild stickleback is evaluated. In addition, the present study is the first to take individual-based differences in body shape, measured using powerful geometric morphometric methods, into account. This allows an examination of how differences in vertebral phenotype are associated with differences in body shape at the level of the individual, providing insight into the dynamics of covariation between adaptively important phenotypic traits within stickleback populations.

**MATERIAL AND METHODS**

**SAMPLING PROCEDURES**

Nine populations were sampled from eight sites, including six lakes and two streams (Table 1). The sites were selected to include ecologically diverse anadromous, benthic, and limnetic stickleback populations from different drainages in the Cook Inlet region of Alaska. Stickleback populations have been studied extensively in this region (Walker, 1997; Heins & Baker, 2008; Gelmond, von Hippel & Christy, 2009; Baker et al., 2010; Park & Bell, 2010; Aguirre & Bell, 2012). Limnetic and benthic populations were allopatric, as is typically the case in Alaska (Walker, 1997; Aguirre & Bell, 2012), and were classified based on divergence in ecological conditions of the lakes and phenotypic characteristics of the fish. The relative littoral area (RLA) is a measure of the percentage of...
littoral habitat available in a lake that has been defined and used elsewhere (Walker, 1997; Aguirre, 2009). It varies between 0 and 100, with higher numbers indicating more littoral or benthic habitat. The benthic and limnetic populations were selected from lakes that differ substantially in RLA. Phenotypic characteristics that have been reported to differ between some or all of the selected benthic and limnetic populations include body shape (Walker, 1997; Aguirre, 2009; Aguirre & Bell, 2012), skull morphology (Willacker et al., 2010), telencephalon morphology (Park & Bell, 2010), dental microwear (Purnell et al., 2006), and opercle shape (Arif, Aguirre & Bell, 2009). Because of the geographical distances among the sites sampled, the resident freshwater benthic and limnetic populations included were most likely independently established by oceanic fish and evolved similar morphological characteristics in parallel.

Fish were collected in June of 2010, except for an anadromous sample collected from Anchor River in 2008. Two populations, an anadromous population and a resident benthic population, were collected at the same site in Mud Lake. Anadromous and resident freshwater populations in Mud lake are easily distinguished based on differences in phenotype (Karve, von Hippel & Bell, 2008; Confer et al., 2012) and do not appear to hybridize (Bell et al., 2010; Drevecky, Falco & Aguirre, 2013), allowing unambiguous identification of resident and anadromous fish. Five to ten unbaited 0.64- and/or 0.32-mm mesh minnow traps were set close to shores overnight. Specimens were anaesthetized with MS-222, fixed in 10% buffered formalin, transferred to 70% ethanol for storage, and stained with Alizarin Red S sensu Bell & Ortí (1994) to facilitate visualization of external bones. Only specimens greater than 32 mm were used to reduce ontogenetic variation.

DATA COLLECTION

Geometric morphometric methods (Zelditch et al., 2004) were used to analyze body shape variation. Sex was identified by internal inspection of gonads. Thirty male and thirty female specimens from each population were individually tagged for analysis of body shape and vertebral morphology. Sample sizes were slightly smaller for Anchor River, Tern Lake and Lynne Lake (Table 1). Specimens infected with Schistocephalus solidus were excluded because the worms may distort body shape. In total, 514 specimens were included in the morphometric analysis. Specimens were straightened (if necessary) using insect pins and were photographed with a 10.3 megapixel Nikon Coolpix P100 digital camera. Two-dimensional coordinates were collected for 16 landmarks digitized on each specimen sensu Aguirre et al. (2008) using TPSDIG, version 2.16 (Rohlf, 2010). The landmark data were aligned using Procrustes superimposition implemented in RELATIVE WARP, version 1.46 (Rohlf, 2008) to eliminate variation related to rotation, translation, and size. All specimens were included in a single alignment for analysis.

The same specimens were X-rayed at the Field Museum of Natural History using an AXR Hot Shot X-ray Machine (Associated X-ray Corporation). Anadromous fish are larger and more heavily armoured than resident freshwater fish, and so slightly different settings were used for these forms to maximize X-ray quality. Anadromous fish were typically X-rayed at 40 kV and 4 Ma for 11 s, whereas

<table>
<thead>
<tr>
<th>Population</th>
<th>Latitude (N), Longitude (W)</th>
<th>N ♂</th>
<th>N ♀</th>
<th>Type</th>
<th>Region</th>
<th>RLA</th>
<th>Surface area (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anchor River</td>
<td>59.773, 151.866</td>
<td>30</td>
<td>29</td>
<td>Anadromous</td>
<td>Kenai</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mud Lake</td>
<td>61.565, 148.947</td>
<td>30</td>
<td>30</td>
<td>Anadromous</td>
<td>Mat-Su</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rabbit Slough</td>
<td>61.534, 149.268</td>
<td>30</td>
<td>30</td>
<td>Anadromous</td>
<td>Mat-Su</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Corcoran</td>
<td>61.573, 149.693</td>
<td>30</td>
<td>30</td>
<td>Resident: Benthic</td>
<td>Mat-Su</td>
<td>100</td>
<td>4.2</td>
</tr>
<tr>
<td>Mud Lake</td>
<td>61.565, 148.947</td>
<td>30</td>
<td>30</td>
<td>Resident: Benthic</td>
<td>Mat-Su</td>
<td>100</td>
<td>26.5</td>
</tr>
<tr>
<td>Tern Lake</td>
<td>60.320, 149.323</td>
<td>22</td>
<td>28</td>
<td>Resident: Benthic</td>
<td>Mat-Su</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>Long Lake</td>
<td>61.576, 149.774</td>
<td>30</td>
<td>30</td>
<td>Resident: Limnetic</td>
<td>Mat-Su</td>
<td>30.21</td>
<td>3.72</td>
</tr>
<tr>
<td>Lynne Lake</td>
<td>61.712, 150.039</td>
<td>21</td>
<td>24</td>
<td>Resident: Limnetic</td>
<td>Mat-Su</td>
<td>51.53</td>
<td>20.16</td>
</tr>
<tr>
<td>Stormy Lake</td>
<td>60.463, 151.032</td>
<td>30</td>
<td>30</td>
<td>Resident: Limnetic</td>
<td>Kenai</td>
<td>32.74</td>
<td>172.60</td>
</tr>
</tbody>
</table>

Latitude and longitude are in decimal degrees of latitude north (N) and longitude west (W). N ♂ and N ♀ are the number of males and females in each sample. Region indicates the area in which the sites are located: Kenai for the Kenai Peninsula and Mat-Su for the Matanuska-Susitna Valley. RLA, relative littoral area, a measure of the benthic and limnetic habitat available in the lake. Larger numbers indicate more benthic habitat.
resident freshwater fish were typically X-rayed at 40 kV and 4 Ma for 8 s. X-rays were scanned at high resolution (1200 p.p.i.) with a HP Scanjet G4100 and transformed to a digital format. The number of abdominal and caudal vertebrae was counted from the digital images (Fig. 1). In most cases, there was a clear transition between abdominal and caudal vertebrae allowing unambiguous classification. Abdominal vertebrae have ribs and lack haemal spines, whereas caudal vertebrae lack ribs and have haemal spines. However, in some specimens, the vertebrae in the region of the transition between the abdominal and caudal regions can be somewhat intermediate in form with haemal arches gradually increasing in size and forming small spine-like structures. Sensu Hubbs & Lagler (2004), we counted vertebrae with haemal arches but lacking spines as abdominal vertebrae and, sensu Swain (1992b), we designated the first vertebra having a definite, long haemal spine as the first caudal vertebra. In stickleback, the first vertebra with a definite haemal spine typically comes into contact or is in close proximity to the first anal pterygiophore (Fig. 1), facilitating classification (Swain, 1992b; Ahn & Gibson, 1999a). In less than 5% of specimens examined in the present study, the last vertebra designated as an abdominal vertebra had a small but visible spine that did not come in contact with the first anal pterygiophore. In all cases in which this occurred, the gap in length between this element and the length of the haemal spine of the first designated caudal vertebra was substantial, with the small spines never reaching approximately half the length of the haemal spine on the first caudal vertebra (based on visual inspection). These vertebrae were classified as abdominal vertebrae in the present study because they appeared more similar to abdominal vertebrae with haemal arches than to caudal vertebrae with fully developed haemal spines. Swain (1992b) used a similar spine length-based approach to designate caudal vertebrae, although he had a more stringent criterion of ≥ 80% length equivalency. The urostyle is not included in vertebral counts.

**Statistical analysis**

Variation in body shape was examined using principal components analysis (PCA) and discriminant function analysis (DFA). The PCA was conducted in RELATIVE WARPS, version 1.46 (Rohlf, 2008), and sample means along the first three PC axes were calculated from individual PC scores and plotted. DFA (as implemented in SPSS, version 11.0.0; SPSS Inc.) was used to examine body shape variation specifically associated with divergence between anadromous and resident, male and female, and benthic and limnetic stickleback. Shape variables (partial warps and uniform component) obtained from a single alignment of all specimens, were used in the DFA. Prior probabilities were obtained from group sizes. Specimens were classified into groups using the discriminant functions obtained and classification rates were calculated using leave-one-out cross-validation. Scores for the three discriminant functions were regressed on body shape using TPSREGR, version 1.36 (Rohlf, 2009) and deformation grids of the predicted shapes associated with extreme values along each DFA axis were created.

Mixed-effects modelling was used to examine whether there were significant associations between vertebral number, ecomorph, sex, and population. A model simplification approach was taken sensu Crawley (2007) in R, version 3.0.2 (The R Project for Statistical Computing). To examine the association between ecomorph, sex and vertebral number, saturated models (models including all interactions) were generated using the LME mixed-effects modelling function with the ML method selected instead of the default RML method to allow testing of models using an analysis of variance-based approach (Crawley, 2007).
In these models, ecomorph and sex were included as fixed factors, whereas population was included as a random factor nested within ecomorph. Each population was given a unique code. The largest-order interactions were excluded from the model in stepwise fashion and tested for significance. Total number of vertebrae, the number of abdominal vertebrae, and the number of caudal vertebrae were analyzed separately. Rare vertebral counts were pooled with neighbouring count classes to avoid having large numbers of zeroes and low count cells in the statistical analysis. Details on pooling of the data are provided in Figure 2. Variation among populations within ecomorphs was also examined separately for each ecomorph using a similar model simplification approach. Models were generated with the GLM function and a Poisson error structure and factors were tested for significance by comparing models with and without the factor of interest.

To examine the specific influence of vertebral phenotype on body shape and generate predicted body shapes for different vertebral phenotype values, body shape was regressed on total number of vertebrae and the ratio of abdominal to caudal vertebrae in TPSREGR, version 1.36 (Rohlf, 2009). The ratio of abdominal and caudal vertebrae indicates the relative proportion of abdominal to caudal vertebrae with larger values indicating a greater proportion of abdominal vertebrae. To account for variation in other variables known to impact upon body shape variation, centroid size (to account for allometry), ecomorph (anadromous, benthic, limnetic), sex, and ecomorph × sex interaction, were also included in the regression model. The significance of each factor was evaluated by running the complete model with and without the factor of interest and testing the significance of the difference in the residual sums of squares matrices as outlined in Rohlf (2009). Finally, the scores from the discriminant function (DF) used to classify benthic and limnetic stickleback from the body shape data were employed as indicators of the relative elongation of individuals along the benthic–limnetic axis. This allowed simplification and visual representation of variation in body shape in relation to vertebral phenotype by summarizing body shape divergence between benthics and limnetics along a single dimension. Individuals with more extreme scores along the discriminant axis were considered to have more extremely divergent body shapes along the benthic–limnetic continuum, whereas individuals with less extreme scores were deemed to be more intermediate in body shape. The relationship between these DF scores and the total number of vertebrae and the ratio of abdominal to caudal vertebrae was examined, as was the distribution of DF scores in benthic and limnetic stickleback by vertebral phenotype.

RESULTS

Total vertebral number varied between 29 and 33 across all samples (Fig. 2; see also Supporting information, Appendix S1, S2). The most common total vertebral count was 31 (57.9% across all samples) and total counts of 29 and 33 vertebrae were relatively rare (<5% combined). The five possible total vertebral counts that stickleback exhibited could be made up from 14 possible combinations of abdominal and caudal vertebrae (abdominal + caudal vertebrae), with the most common being 15 + 16 (43.9%), 15 + 17 (17.1%), and 14 + 17 (10.7%). The numbers of abdominal and caudal vertebrae were negatively correlated ($r = -0.46, N = 514, P < 0.001$), indicating that vertebral homeosis (the transformation of vertebral identities) is common in the axial skeleton of stickleback. This held both within ecomorphs ($r = -0.37, -0.32, and -0.57, P < 0.05$, for anadromous, limnetic, and benthic fish) and when sexes were examined separately ($r = -0.45$ and $-0.56, P < 0.001$, for males and females, respectively).

Body shape varied substantially among the stickleback populations sampled in accordance with expectations from previous studies. The first three PC axes accounted for 67.5% of the variance in body shape and were biologically interpretable according to the scatter of specimens in the morphospace. PC I was associated with divergence between anadromous and resident lake stickleback, PC II was associated with variation between males and females, and PC III was associated with divergence between benthics and limnetics (Fig. 3). The DFA yielded similar results and successful classification rates were high at >95% (Fig. 4, Table 2). Body shape divergence among ecomorphs and between sexes has been described previously (Walker, 1997; Kitano, Mori & Peichel, 2007; Aguirre et al., 2008; Aguirre & Bell, 2012), and so we limit our description below to aspects relevant to vertebral variation.

Differences in vertebral number among sexes, ecomorphs, and populations within ecomorphs were significant. These differences translated to significant associations between body shape and vertebral number. Even when accounting for body size variation, ecomorph, and sex, the total number of vertebrae and the ratio of abdominal to caudal vertebrae was associated with a significant portion of the variation in body shape among stickleback (Table 3).

Sexual dimorphism in the body shape of stickleback (Figs 3, 4) was accompanied by corresponding differences in vertebral number. Total vertebral number did not differ significantly between sexes, although the number of abdominal and caudal vertebrae did (Table 4), and this difference held when examined separately by ecomorph (see Supporting information,
Figure 2. Number of abdominal (A, B), caudal (C, D), and total (E, F) vertebrae pooled by ecomorph. A, C, E include all vertebral counts. B, D, F illustrate how rare vertebral counts were pooled with neighbouring count classes for statistical analysis to avoid a large number of zeros and low count cells. Variation among populations within ecomorphs was also analyzed separately for each ecomorph and vertebral number was pooled into classes, as illustrated above, except that anadromous fish did not have counts of 14 caudal vertebrae, benthics did not have total counts of 33 or caudal counts of 18, and limnetics did not have total counts of 29 or caudal counts of 14; thus, pooling of these classes was unnecessary. In addition, because of differences in count frequencies, abdominal counts were pooled in two classes (13–14 and 15–16) and caudal counts were pooled into classes of 15–16, 17, and 18 for limnetics.

Appendix S3). Females had a mean ± SEM of 0.31 ± 0.04 more abdominal vertebrae, whereas males had 0.41 ± 0.07 more caudal vertebrae (see Supporting information, Appendix S2). Although the magnitude of this difference was not large, the mean number of abdominal vertebrae was greater in females (and, consequently, the mean number of caudal vertebrae was greater in males) in all nine populations examined (Fig. 5). The expansion of the abdominal region in females (Fig. 4) is thus associated with an increase in vertebral number in this region.

Total vertebral number differed significantly among ecomorphs (Table 4). The anadromous populations were generally homogeneous in mean total vertebral number (with the exception of Rabbit Slough females; Fig. 6) and exhibited all five total vertebral number phenotypes. The resident freshwater stickleback exhibited a more limited range of total vertebral number; no limnetic fish had 29 vertebrae and no benthic fish had 33 vertebrae (see Supporting information, Appendix S1). Two of the three limnetic populations (Stormy and Long) had more vertebrae than populations of the other ecomorphs (Fig. 6). These differences in vertebral number generally corresponded to body shape differences among ecomorphs. Fish with a greater number of total vertebrae across all samples tended to be more elongate than fishes with a lower total number of vertebrae (Fig. 7A, B). The head appeared to be relatively smaller and the dorsal and anal fins and the caudal peduncle were longer in fish with more vertebrae. Total vertebral number was also correlated with scores along the benthic–limnetic DF axis ($r = 0.30$, $P < 0.001$), indicating that fishes at the extremes of the benthic–limnetic axis tended to have fewer total vertebrae (more benthic-like) and more total vertebrae (more limnetic-like). Moreover, this held within ecomorphs for the limnetics ($r = 0.32$, $P < 0.001$) and benthics ($r = 0.19$, $P < 0.001$), indicating that variation in body shape along the benthic–limnetic axis was associated with total vertebral number even within ecomorphs.

The number of abdominal and caudal vertebrae also differed significantly among ecomorphs in ways that were consistent with the observed body shape differences, with some significant variation among populations. Across all samples, fishes with greater

Figure 3. Principal components analysis (PCA) of body shape data. Symbols indicate sample means. Percentages next to axis labels indicate the percentage of variation explained by each axis. PC I was associated with divergence between anadromous and resident freshwater populations, PC II was associated with sexual dimorphism, and PC III was associated with divergence between benthic and limnetic populations.
ratios of abdominal to caudal vertebrae (a greater proportion of abdominal vertebrae) tended to have an expanded abdominal region, whereas fish with lower ratios (a greater proportion of caudal vertebrae) tended to have an expanded caudal region (Fig. 7C, D). Although the contribution of vertebral length was not quantified and may be contributing as well, these data indicate that variation in vertebral number contributes to variation in body shape. Most of the resident freshwater populations had a lower mean number of abdominal vertebrae than the anadromous populations, which were fairly homogeneous (Fig. 5). This is consistent with the larger abdominal region of the body seen in anadromous fish and the expanded caudal body region seen in resident freshwater fish. Moreover, the ratio of abdominal to caudal vertebrae was significantly negatively correlated with scores along the benthic–limnetic DF axis in resident

Table 2. Percentage classification of stickleback into groups by discriminant function analysis of body shape (leave-one-out cross-validation)

<table>
<thead>
<tr>
<th>Original</th>
<th>Classified as:</th>
<th>Original</th>
<th>Anadromous</th>
<th>Resident</th>
<th>Male</th>
<th>Female</th>
<th>Benthic</th>
<th>Limnetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anadromous male</td>
<td>100</td>
<td>0</td>
<td>97.8</td>
<td>2.2</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anadromous female</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benthic male</td>
<td>0</td>
<td>100</td>
<td>95.1</td>
<td>4.9</td>
<td>98.8</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benthic female</td>
<td>0</td>
<td>100</td>
<td>3.4</td>
<td>96.6</td>
<td>94.3</td>
<td>5.7</td>
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<td>Limnetic male</td>
<td>0</td>
<td>100</td>
<td>98.8</td>
<td>1.2</td>
<td>0</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limnetic female</td>
<td>0</td>
<td>100</td>
<td>1.2</td>
<td>98.8</td>
<td>2.4</td>
<td>97.6</td>
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</tr>
</tbody>
</table>

Correct classification percentages are indicated in bold.

Figure 4. Predicted body shape divergence among stickleback from regression of body shape variables on discriminant function scores. A, predicted shape for anadromous stickleback. B, lake resident stickleback. C, male stickleback. D, female stickleback. E, benthic stickleback. F, limnetic stickleback. Eye, spine, and fin location and sizes are approximate. Body shape variation exaggerated by approximately two-fold to facilitate visualization.
freshwater stickleback \( (r = -0.30, P < 0.001) \), indicating that fish with a greater proportion of abdominal vertebrae tended to be more benthic-like and fish with a greater proportion of caudal vertebrae tended to be more limnetic-like. This held for the two limnetic populations in Stormy Lake and Long Lake, in which the greater total vertebral number seen appeared to be driven primarily by an increase in caudal vertebrae. This was most striking in the Stormy Lake population, which had more caudal vertebrae than all other populations examined (Fig. 5). Of 34 fish exhibiting the highest caudal vertebral counts (18 caudal vertebrae), 25 were from Stormy. Of the 18 fish exhibiting total vertebral counts of 33, 16 were from Stormy. Within ecomorphs, the number of abdominal vertebrae differed significantly only among benthic populations and this appeared to be driven largely by the Tern Lake population (see below), whereas differences among populations in the number of caudal vertebrae were significant in all three ecomorphs (see Supporting information, Appendix S3), highlighting the presence of substantial variation in vertebral number in this region. The relationship between the ratio of abdominal to caudal vertebrae and scores along the benthic–limnetic DF axis did not hold within ecomorphs \( (r = -0.12, r = -0.07, P > 0.05, \) for limnetics and benthics, respectively).

There was significant variation among populations in vertebral phenotypes, as well as a substantial amount of variation in body shape along the benthic–limnetic axis that was independent of differences in vertebral number. For example, the benthic Tern Lake population had a substantially greater mean number of abdominal vertebrae than all other populations including the other two benthic populations with similar body shapes and anadromous stickleback with expanded anterior body regions. In addition, the limnetic Lynne Lake population had the lowest mean total number of vertebrae in the study (most fish had 30 or 31 vertebrae) and a corresponding low mean number of caudal vertebrae despite having a body shape similar to the other two limnetic populations. Plotting scores along the benthic–limnetic DF axis against vertebral phenotypes, there was broad scatter of DF scores among individuals with the same vertebral phenotype (Fig. 8). This was true even among individuals of the same sex and from the same ecomorph and population, although it was most notable among individuals from different ecomorphs. Benthic and limnetic stickleback with the same number of abdominal and caudal vertebrae differed substantially in body shape along most of the spectrum of vertebral phenotypes observed in the present study. Thus, substantial divergence in body shape could be accomplished without modification of vertebral number.

**DISCUSSION**

Consistent with expectations, we found a significant association between body shape and vertebral number, variation in the proportion of abdominal and caudal vertebrae, and significant sexual dimorphism in vertebral number. There was also substantial variation among populations within ecomorphs and among individuals within populations, indicating that similarity in external morphology may mask significant variation in the vertebral column and that changes in body form may be associated with different patterns of change in the axial skeleton. Although more research is needed to broaden our understanding of the underlying mechanisms and achieve greater

| Table 3. Influence of centroid size, ecomorph (anadromous, benthic, limnetic), sex, ecomorph × sex interaction, total vertebral number (Total vert), and the ratio of abdominal to caudal vertebrae (Abd : Caud) on body shape |
|-----------------|--------|-------|-------|-------|
| Factor          | Wilks’ \( \lambda \) | \( F \) | d.f. 1 | d.f. 2 |
| Centroid size   | 0.64   | 9.85  | 28    | 478   |
| Ecomorph        | 0.08   | 43.38 | 56    | 956   |
| Sex             | 0.21   | 62.58 | 28    | 478   |
| Ecomorph × Sex  | 0.65   | 4.17  | 56    | 956   |
| Total vert      | 0.76   | 5.40  | 28    | 478   |
| Abd : Caud      | 0.80   | 4.31  | 28    | 478   |

All factors were significant \( (P < 0.001) \).

| Table 4. Mixed-effects model analysis of the association between vertebral number, ecomorph, and sex, taking population into account |
|-----------------|--------|--------|-------|
| Factor          | d.f.   | Likelihood ratio | \( P \) |
| Total vertebrae |         |                   |       |
| Ecomorph (Population) | 4      | 16.99            | 0.002 |
| Sex             | 2      | 0.66             | 0.718 |
| Ecomorph × Sex  | 4      | 0.51             | 0.973 |
| Abdominal vertebrae |       |                   |       |
| Ecomorph (Population) | 4      | 17.24            | 0.002 |
| Sex             | 2      | 13.70            | 0.001 |
| Ecomorph × Sex  | 4      | 5.48             | 0.242 |
| Caudal vertebrae |         |                   |       |
| Ecomorph (Population) | 4      | 27.12            | < 0.001 |
| Sex             | 2      | 10.79            | 0.005 |
| Ecomorph × Sex  | 4      | 0.45             | 0.978 |

Ecomorph and sex are fixed factors, whereas population is a random factor nested within ecomorph. Significance of fixed factors was assessed through a model simplification approach.

perspective on the functional consequences of the differences in vertebral phenotypes, our findings highlight the potential of the threespine stickleback for studying the origin of evolutionary diversity in the axial skeleton.

SEXUAL DIMORPHISM
The most consistent pattern in the data was the sexual dimorphism in the number of abdominal and caudal vertebrae. Females had a significantly greater mean number of abdominal vertebrae and males had a corresponding greater mean number of caudal vertebrae. The magnitude of the difference was not large, although the pattern was similar in every population across all ecomorphs, including the samples of the ancestral anadromous form, suggesting that this is a broadly conserved trait. The results of our analysis of sexual dimorphism in body shape, as well as data from previous studies (Aguirre et al., 2008), indicate

**Figure 5.** Mean number of abdominal and caudal vertebrae by population and sex. Ellipses connect male and female means by population except for the anadromous populations, which are enclosed in a single ellipse because of their relative homogeneity.

**Figure 6.** Mean total number of vertebrae plotted by population and sex. The x-axis indicates population type (anadromous, limnetic or benthic).
that female stickleback have an expanded abdominal region relative to males. An increase in the number of abdominal vertebrae thus appears to contribute to this expansion of the abdomen in females. It remains to be seen whether changes in vertebral length are also contributing. The difference in vertebral phenotypes between sexes appears to be achieved largely by vertebral homeosis (Ahn, 1998) because total vertebral number did not differ significantly between sexes. Lindsey (1962) reported similar results for threespine stickleback previously, and a greater number of abdominal vertebrae in females has been reported in other fish species (Hart & McHugh, 1944; Hastings, 1991). Lindsey (1962, 1975) suggested that this is an adaptation for greater egg capacity in the abdomen. If true, it would likely be more pronounced

**Figure 7.** Predicted body shapes for extreme vertebral phenotypes from regression of body shape on total vertebral number and the ratio of abdominal to caudal vertebrae. A, predicted body shape for fish with low total vertebral counts. B, predicted body shape for fish with high total vertebral counts. C, predicted body shape for fish with a greater proportion of abdominal vertebrae. D, predicted body shape for fish with a greater proportion of caudal vertebrae. Deformation grids are exaggerated five-fold. Eyes, fins, and spines were drawn manually and are approximate in size and location.

**Figure 8.** Variation of resident lake stickleback along the benthic–limnetic body shape axis (as inferred from discriminant function scores) plotted by vertebral phenotype and sex. Numbers along the abscissa represent abdominal/caudal vertebral numbers (13/16–16/17), with total vertebral number (29–33) listed below. A, males. B, females. Triangles are limnetic and squares are benthic populations. Colours identify individual populations.
in species in which gravid females have tightly packed eggs in a conspicuously swollen abdomen.

As was the case in the present study, Lindsey (1962) and Alho et al. (2011) found no significant difference between male and female total vertebral number (they did not distinguish between abdominal and caudal vertebrae). This differs from the results of Reimchen & Nelson (1987), who found significantly higher total vertebral counts (+0.18 vertebrae) in male stickleback from Drizzle Lake on the Queen Charlotte Islands, Canada. A survey of ten other populations from the area confirmed that male stickleback from the region had more vertebrae than females (0.3 more on average). Although not statistically significant, males had higher mean vertebral counts than females in six of the nine populations that we sampled. Averaged across populations, the difference in the present study was +0.11 vertebrae in males (range −0.23 to +0.50), which is comparable to the difference of +0.18 for Drizzle Lake males.

**VERTEBRAL VARIATION AMONG ECOMORPHS AND POPULATIONS**

The highest vertebral counts occurred in limnetic populations, which is consistent with previous findings on the impacts of body elongation on the vertebral column of fishes at broader taxonomic ranks (Ward & Brainerd, 2007; Ward & Mehta, 2010), as well as some previous studies in stickleback (Reimchen & Nelson, 1987; Ahn, 1998). In particular, the limnetic Stormy Lake population had a substantially greater mean number of vertebrae than all other populations and the limnetic Long Lake population had the second highest mean number of vertebrae (Fig. 6). Thus, regardless of variation in vertebral lengths, the results of the present study indicate that vertebral number contributes significantly to variation in body form among ecomorphs. The increase in vertebral number in these limnetic populations was largely the result of an increase in caudal vertebrae because the number of abdominal vertebrae was similar in most freshwater populations (with the exception of the Tern Lake population) and less than that of the anadromous populations. Limnetics in this and previous studies of body shape variation (Walker & Bell, 2000; Aguirre et al., 2008; Aguirre, 2009) have an expanded caudal region relative to deeper bodied benthic populations. Given the increase in total vertebral number, elongation in at least some limnetic populations is associated with an addition of vertebrae in the caudal region, and not just vertebral homeosis as was the case with expansion of the abdominal region in female stickleback. This association between vertebral number and body elongation held within ecomorphs as well and was strongest in the limnetic ecomorph, indicating that there is significant variation in body shape among individuals associated with differences in vertebral counts.

Vertebral phenotypes differed substantially among populations of the same ecomorph. Elongation of limnetics was not always associated with an increase in vertebral number. One of the elongate limnetic populations, the Lynne Lake population, had the lowest mean number of vertebrae in the present study and one of the lowest mean numbers of caudal vertebrae. Presumably, the Lynne Lake population achieves an elongate body form with an increase in the length of vertebrae per unit body size rather than an increase in vertebral number. The benthic populations also exhibited heterogeneity in vertebral phenotypes. Most notably, the Tern Lake population had a much higher mean number of abdominal vertebrae than all other populations surveyed. Although the number of caudal vertebrae was slightly lower than in the other benthic populations suggesting the possibility of some vertebral homeosis, the Tern Lake population had the third highest mean number of total vertebrae behind only the limnetic Stormy and Long Lake populations, suggesting that Tern stickleback add vertebrae in the abdominal region. In addition, the body shape of stickleback exhibiting the same abdominal and caudal vertebral counts, including the highly divergent benthics and limnetics, could differ remarkably (Fig. 8). The occurrence of individuals with similar body shapes but different vertebral phenotypes, and vice versa, indicates that there is substantial flexibility in the relationship between the axial skeleton and body shape in stickleback.

Differences in patterns of modification of the axial skeleton have been documented previously. For example, Ahn (1998) found that elongation was often but not always associated with an increase in vertebral number in the stickleback populations that he examined. Ward & Mehta (2010) reviewed body elongation in fishes and indicated that, although elongation is typically associated with an increase in vertebral number, some species such as *Sphyraena barracuda*, achieve elongate bodies by increasing vertebral length. Similarly, different clades of plethodontid salamanders that have independently evolved body elongation can achieve this elongation in different ways; some increase the number of vertebrae, whereas others increase the length of vertebrae (Wake, Wake & Specht, 2011). Thus, the occurrence of different vertebral phenotypes underlying similar body shapes may not be uncommon in vertebrate lineages that independently evolve body elongation. Why these different trajectories are taken is unclear, as are their functional consequences.
Although the addition of extra vertebrae appears to contribute to divergence in vertebral phenotypes among limnetic and benthic populations, vertebral homeosis also likely plays a role. Higher counts of abdominal or caudal vertebrae often appear to be often by transforming vertebral identity rather than by the addition of vertebrae in the abdominal and caudal regions. Vertebral number in the abdominal and caudal regions can vary independently in fishes and appears to be regulated by different developmental systems (Ward & Mehta, 2010), and so this transformation of vertebral identity is likely associated with changes in gene expression along the body axis. Identifying the genes involved and how their expression is modified is a potential direction for future research.

FUNCTIONAL SIGNIFICANCE OF VARIATION IN VERTEBRAL PHENOTYPES

More elongate, streamlined bodies are considered to be favoured in limnetic populations because they reduce drag associated with prolonged swimming in open water (Walker & Bell, 2000; Spoljaric & Reimchen, 2007). It is unclear why an expansion of the caudal region would specifically be favoured in limnetics. Increases in the length of the median fin bases increase hydrodynamic efficiency because a deeper caudal region increases thrust during swimming. Fins provide an increase in the depth of the caudal region with a corresponding increase in thrust but less drag than would result from increasing the depth of posterior part of the body (Walker, 1997; Blake et al., 2005). The expansion of the caudal region may thus be associated with selection for an expansion of median fin length in limnetics. In stickleback, the position of the anal fin is also correlated with the transition to caudal vertebrae along the body axis because the haemal spine of the first caudal vertebra typically interlocks with the first anal pterygiophore. Thus, both the length and positioning of median fins may play a role.

The ratio of abdominal to caudal vertebrae has also been shown to be functionally important in threespine stickleback. Swain (1992a, 1992b) examined fast-start performance in young threespine stickleback reared in the laboratory and found that the ratio of abdominal to caudal vertebrae had a significant effect on fast-start performance. A study of prolonged swimming and fast-start performance of anadromous, benthic, and limnetic stickleback performed by Blake et al. (2005) found that benthic and limnetic stickleback had greater fast-start velocities than anadromous stickleback but similar performances to one another, despite the differences in body shape. Given the deeper bodies of benthics, fast-start performance was expected to be greater in benthics than in limnetics. Blake et al. (2005) highlighted the deep, posteriorly placed median fins of limnetics as a potential explanation for their over-performance. However, the results reported by Swain (1992a, b) raise the possibility that differences in vertebral phenotypes may also be at play. Consistent with this possibility, Brainerd & Patek (1998) found that lower vertebral number was correlated with lower C-start curvature across several tetraodontiform species varying in vertebral number, suggesting that reductions in vertebral number may impair escape performance. In as much as an increase in vertebral number increases the flexibility of the body, more vertebrae may be favoured by selection for enhanced fast-start performance in some limnetic populations.

FUTURE DIRECTIONS

Much remains to be learned about vertebral variation in ecologically and morphologically divergent threespine stickleback. Although previous studies have shown that there is a signifcant genetic component to variation in stickleback vertebral number (Ahn & Gibson, 1999a; Hermida et al., 2002; Alho et al., 2011), an important task will be to quantify how much of the variation documented in the present study is a result of environmental factors. The genomic resources that are available, as well as improving knowledge of the development of the vertebrate body axis, make the threespine stickleback a promising system in which to study how genes and developmental pathways associated with vertebral development are modified in populations adapting to different ecological niches (Ahn & Gibson, 1999b, c). Along these lines Berner et al. (2014) recently identified candidate genes associated with variation in vertebral number between European lake and stream stickleback populations, providing a promising future line of inquiry for studies on the genetic basis of vertebral evolution in stickleback. Another promising direction is to examine whether modification of vertebral length is significantly associated with body shape evolution in stickleback. The amount of body shape variation that appeared unrelated to vertebral number was substantial, and variation in vertebral length is an obvious candidate to account for some of this unexplained variation. There was also substantial variation in vertebral phenotypes among individuals within populations and much more work is needed on understanding what causes this variation. Moreover, examining whether differences in vertebral phenotype impact upon swimming/escape performance in individuals with similar body shapes is a particularly interesting research direction to pursue.
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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Vertebral phenotype frequencies by population and sex.

Appendix S2. Mean abdominal and caudal vertebral number and abdominal to caudal ratios of populations surveyed (with minimum and maximum values in parenthesis). Anch Riv, Anchor River; Mud An, Mud anadromous; RS, Rabbit Slough; Corc, Corcoran Lake; Mud Res, resident lake population sampled from Mud Lake. The mean ± SEM at the bottom of the table are calculated from sample means.

Appendix S3. Log-linear modelling analysis of vertebral number variation by population (Pop) and sex within ecomorphs. Tests were run separately on abdominal and caudal vertebrae. DF, degrees of freedom; SS, sum of squares.