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Developmental timing differences underlie armor loss across threespine stickleback populations

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Comparing ontogenetic patterns within a well-described evolutionary context aids in inferring mechanisms of change, including heterochronies or deletion of developmental pathways. Because selection acts on phenotypes throughout ontogeny, any within-taxon developmental variation has implications for evolvability. We compare ontogenetic order and timing of locomotion and defensive traits in three populations of threespine stickleback that have evolutionarily divergent adult forms. This analysis adds to the growing understanding of developmental genetic mechanisms of adaptive change in this evolutionary model species by delineating when chondrogenesis and osteogenesis in two derived populations begin to deviate from the developmental pattern in their immediate ancestors. We found that differences in adult defensive morphologies arise through abolished or delayed initiation of these traits rather than via an overall heterochronic shift, that intrapopulation ontogenetic variation is increased for some derived traits, and that altered armor developmental timing differentiates the derived populations from each other despite parallels in adult lateral plate armor phenotypes. We found that changes in ossified elements of the pelvic armor are linked to delayed and incomplete development of an early-forming pelvic cartilage, and that this disruption likely presages the variable pelvic vestiges documented in many derived populations.

1 | INTRODUCTION

Analyses that compare ontogenies within a microevolutionary context can identify developmental differences that give rise to differentially adapted morphologies. When paired with what is known about the genetic basis of homologous traits from model species, such a "micro-evo-devo" approach (Johnson, 2007) can inform how known genetic variation gives rise to phenotypic variation (Gilbert, Bosch, & Ledon-Rettig, 2015; Hallgrimsson et al., 2012; Streelman, 2013). These data can also help identify possible developmental biases affecting evolutionary trajectories (Arthur, 2001, 2011; Hallgrimsson et al. 2012; Hendrikse, Parsons, & Hallgrimsson, 2007) and can suggest likely gene pathways that might have been affected by selection (Brakefield, 2006; Cresko, McGuigan, Phillips, & Postlethwait, 2007; Psujek & Beer, 2008).

Threespine stickleback fish have become a model for the study of vertebrate microevolution (Bell & Foster, 1994; Jamniczky, Barry, & Rogers, 2015; Marchinko & Schluter, 2007; Peichel & Marques, 2017; Walker & Bell, 2000). A sequenced reference genome and amenability for developmental studies in the lab have made stickleback a key resource for comparative evo-devo studies across divergent fish lineages (Cresko et al., 2007; Jamniczky et al., 2015; Kimmel et al., 2005, 2012; Wark & Peichel, 2010). An attribute of particular interest in this widely distributed species is that behavioral, physiological, and morphological adaptive evolution has occurred wherever stickleback from the sea have invaded freshwater streams and ponds, and has likely recurred

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often (Bell & Aguirre, 2013; Schluter & Conte, 2009) since the species began at least 10 million years ago (Bell, 2009; Bell, Stewart, & Park, 2009). Such change in stickleback can take place even on the order of decades (Hagen & Gilbertson, 1973; Klepaker, 1993; Bell, 2001; Bell & Aguirre, 2013; Bell, Aguirre, Buck, & Wainwright, 2004; Bell et al., 2016; Lescak et al., 2015; Terekhanova et al., 2014). The different demands of alternative habitats for predation survival, locomotion, and feeding are reflected in modified behavioral, defensive armor, fin, body shape, and cranial traits (Bell & Foster, 1994; Hagen & Gilbertson, 1972; Huntingford, Wright, & Tierney, 2004; Kimmel et al., 2012; Reimchen, 1994; Walker, 1997; Walker & Bell, 2000; Wund, Baker, Golub, & Foster, 2015).

Remarkably similar morphologies seen in independently derived freshwater stickleback populations strewn across the northern hemisphere have sparked intensive efforts to understand the genomic basis of this parallel evolution (Hohenlohe et al., 2010; Jones et al., 2012; Roesti, Gavrilets, Hendry, Salzburger, & Berner, 2014), and a primary goal has been to uncover the underlying genes (Miller et al., 2014; Peichel et al., 2001; Peichel & Marques, 2017). Mapping of quantitative trait loci (QTL) using controlled genetic crosses in the lab (Colosimo et al., 2004; Cresko et al., 2004; Miller et al. 2014; Shapiro et al., 2004), genome scans that uncover patterns of allele frequency divergence in natural populations (Hohenlohe et al., 2010; Marques et al., 2016; Roesti, Hendry, Salzburger, & Berner, 2012), and genome re-sequencing (Jones et al., 2012), have helped delineate genomic regions that underlie divergence of freshwater and marine stickleback traits such as body shape (Albert et al., 2008), craniofacial elements (Cleves et al., 2014; Glazer, Cleves, Erickson, Lam, & Miller, 2014), pigmentation (Malek, Boughman, Dworkin, & Peichel, 2012; Miller et al., 2007), behavior (Greenwood et al., 2015) and, among the most conspicuous features, the stickleback's bony armor (Colosimo et al., 2005; Cresko et al., 2004; Shapiro et al., 2004). Major QTL for defensive lateral plates and pelvic structure variation have been identified and found to be largely independent (Chan et al., 2010; Cole, Tanaka, Prescott, & Tickle, 2003; Colosimo et al., 2004; Cresko et al., 2004; Colosimo et al., 2005; Shapiro et al., 2004).

For example, QTL mapping helped confirm a major locus for the loss of the defensive pelvic spine seen in many freshwater populations (Cole et al., 2003; Cresko et al., 2004; Shapiro et al., 2004), and subsequent embryological and transgenic approaches showed that in this genomic region *pitx1*, a gene known to be important for vertebrate hindlimb development (Marcil, 2003), displays altered regulation in developing freshwater versus marine stickleback (Chan et al., 2010). While this is an exceptional success in the search for the "large effect" developmental genetic underpinnings of evolutionary change — and one from which inferences have been extrapolated to underly the independent origin of "low pelvic" stickleback populations worldwide (Bell, Khalef, & Travis, 2006; Coyle, Huntingford, & Peichel, 2007)—genetic mapping also uncovered other loci of smaller effect that may be affected by selection themselves (Cresko et al., 2004; Peichel et al., 2001; Shapiro et al., 2004). How these genetic effects, both large and small, are manifested during development is still largely unknown, and QTL often span a region with multiple possible genes of interest. Thus, having a robust comparative developmental study across differentially adapted stickleback populations can put these results into context and help guide the search for gene candidates.

To add critical ontogenetic description of skeletal traits that evolutionarily diverge as stickleback adapt to new habitats, we report here on the post-embryonic development of bone and cartilage elements, such as defensive armor traits, as they take shape in stickleback from the ancestral, marine lineage compared with fish from two independently derived freshwater-adapted populations. We asked when the ontogenies of freshwater stickleback begin to diverge from the inferred ancestral program, and whether fish from different freshwater populations that have evolved parallel adult phenotypes arrive at those morphologies through identical developmental paths. Hampering an understanding of divergent stickleback traits, most descriptions of teleost morphogenesis from models like zebrafish and medaka, as well as from stickleback itself, focus only on embryonic stages (Iwamatsu, 2004; Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Swarup, 1958). However, many skeletal elements develop post-embryonically in teleosts, including structures such as the pelvic fins and scales that share a deep homology with stickleback defensive armor traits, and modifications of these later stages might be particularly important for ecologically important adult traits. We used lab rearing to minimize environmental variation experienced during growth, and focused our comparative analyses—from hatching until a stage in which all major bones and cartilages have formed—on well-studied traits of adaptive significance (Bell, 1981; Igarashi, 1970; Swarup, 1958). These detailed observations of post-embryonic skeletal development that compare morphologically divergent populations can provide novel information about vertebrate development, but also explore how core developmental programs can change in an "evolutionary mutant" model (Albertson, Cresko, & Postlethwait, 2009).

2 | METHODS

2.1 | Collections and description of the stickleback populations

Threespine stickleback populations in the Matanuska-Susitna Borough near Anchorage, Alaska are particularly well suited for studies of adaptive traits (Bell, Francis, & Havens, 1985;

Bell & Orti, 1994; Cresko et al., 2004). Over the last approximately 15,000 years after glaciers began receding, stickleback from the sea, which use much of the low-lying freshwater habitat as spawning grounds, have independently given rise to thousands of freshwater-adapted populations spread throughout this region (Bell & Foster, 1994). Direct descendants of these marine and freshwater populations now exist allowing us to investigate specific biological aspects of adaptive evolution.

We tracked the developmental sequence and timing of the initiation of bones and cartilage in one ancestral marine and two derived freshwater populations from this region. As an ancestor ("ANC") surrogate, marine fish from Rabbit Slough (N 61.5595, W 149.2583) were used to establish stocks in the lab in 2003, and these had been in the lab for two generations at the time of this study. Fish from the ANC population have robust bony armor, including a full complement of lateral plates and a large pelvic structure (Figure 1). Two independently derived ("DER") freshwater-adapted populations were sampled; one from Mud Lake (DER1) (N 61.563, W 148.9486), in which fish have evolutionarily lost most of the lateral plates but have a complete (but often smaller than ANC's) pelvic structure (Bell et al., 1985; Bell & Harris, 1985; Bell, 1987; Bell & Orti, 1994) (Figure 1a), and Boot Lake (DER2) (N 61.7167, W 149.1167), in which fish retain only a small subset of lateral plates but also have a very reduced or completely absent pelvic structure (Figure 1a). Laboratory lines were established from Mud Lake fish collected in 2004 and from Boot Lake fish collected in 2000. At the time of this study, these stocks had been cultured in the lab for one and four generations, respectively.

2.2 | Crosses and husbandry

Crosses were made within each genetic line (ANC, DER1, and DER2), and the offspring were grown using standard husbandry procedures developed in the Cresko Lab (Cresko et al., 2004). All protocols and procedures adhere to University of Oregon IACUC approved methods for the ethical care and use of animals. Briefly, after embryos had developed two cells, they were cleaned with embryo medium (EM), consisting of 4 ppt artificial sea water (Instant Ocean) dissolved in nanopure water. Wild juvenile ANC may normally experience a relatively wide range of salinities, as they develop in a slough with tidal flux. DER1 and two juvenile fish experience a more stable, low-salinity environment (e.g., Benolkin, 2011). We chose a salinity of 4 ppt as our lines from these populations had high reproductive success and low embryonic mortality at this salinity, and these conditions deter pathogens. Groups of 20 embryos were placed in individual $26 \times 100\,mm^2$ Petri dishes filled with ~75 ml of EM, and raised in an incubator maintained constantly at 20°C. At 2 and 6 days post-fertilization (dpf), any non-developing embryos were removed and 100% of EM was changed. Rearing continued in this manner until 9 dpf, at which point the fry had hatched and their yolks had been absorbed. Fry were placed in a recirculating aquaculture system at a density of 20 fish per 2.8 L tank. Water temperature was maintained at 20°C, and a salinity of 4 ppt was maintained with Instant Ocean. Fish were fed ad libitum with live *Artemia* (brine shrimp) nauplii and dry food (Ziegler AP100 larval food) twice per day.

2.3 | Sampling and cartilage and bone staining

To determine the critical time points in initiation of cartilage and bone in post-embryonic stickleback, we extensively sampled ANC fish each day from hatching (7 dpf) until 50 dpf when all major cartilages and bone elements are present (Swarup, 1958). We found that, in ANC, the cartilages and bone of the traits examined initiate between 15 and 30 dpf. Therefore, we concentrated our sampling of the derived populations during this window of time (Supplementary Figure S1).

On average, eight fish were sampled each day for ANC (357 total) and six fish were sampled each day for the two derived populations (197 from DER1 and 123 from DER2). Two separate families, on average, were sampled per day for each population, but depth of sampling and distribution across families varied per day (Supplementary Figure S1). Once collected, fish were euthanized with MS-222, fixed in 2% PFA, and then simultaneously stained for cartilage and bone as described in Walker and Kimmel (2007).

2.4 | Phenotyping

Age, standard length, the total number of lateral plates, the presence or absence of foramina in spine supporting structures, and the presence or absence of cartilage and ossification of the elements of the medial and caudal fins, the dorsal spines, and pelvic complex were recorded (Figure 1b–d). Stained fish were imaged along with size standards using a Nikon CoolPix5000 digital camera mounted on a Leica MZ6 stereomicroscope. Standard length was measured using ImageJ 1.46f software (Schneider, Rasband, & Eliceiri, 2012). Using a dissecting microscope the presence or absence of cartilages and ossification of bone was scored. Positive alizarin staining in the expected place of a lateral plate in a body segment was counted as a plate, regardless of its size. Dorsal spine and pelvic structure foramina were scored as present when the anterior and posterior edges of the supporting structure approached each other and an obvious hole could be discerned. The initiation of cartilage and ossification of bone was scored as present when tissues stained either blue or red, respectively, and displayed the

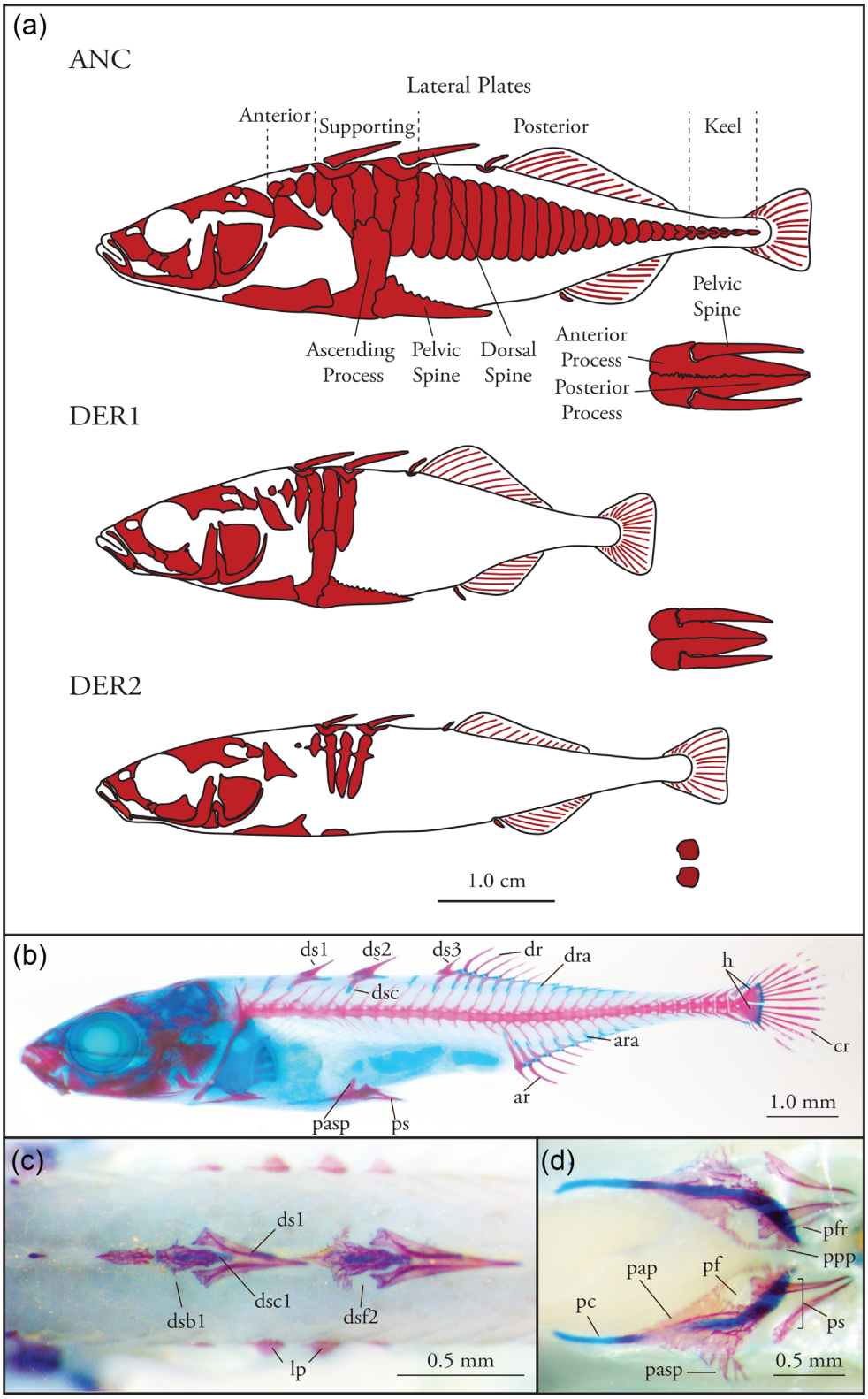

FIGURE 1 Comparative morphology of fish from three populations and the developing skeleton of the ancestral (ANC) form. (a) Lateral views of representative fish with ventral view (inset) of the pelvic structure of each. Superficial bones are in red. Classes of lateral plates and elements of the pelvic structure are labeled (please note that boundaries of lateral plate classes are loosely defined here and that there is overlap between the classes). (b–d) Lateral (b), dorsal (c), and ventral (d) views of a developing ANC fish at 11 mm SL (24 dpf) with bone stained by alizarin red and cartilage stained by alcian blue. **ar**, anal rays; **ara**, anal radials; **cr**, caudal rays; **dra**, dorsal radials; **dr**, dorsal rays; **ds1-3**, dorsal spine; **dsc1-3**, dorsal spine cartilage; **dsf1-3**, dorsal spine foramen; **dsb1-3**, dorsal spine basal plates; **h**, hypural; **lp**, lateral plates; **pap**, pelvic anterior process; **pasp**, pelvic ascending process; **pc**, pelvic cartilage; **pf**, pelvic foramen; **pfr**, pelvic fin ray; **ppp**, pelvic posterior process; **ps**, pelvic spine

correct gross cell morphology for either cartilage, ossifying cartilage, or ossifying dermal or membrane bone.

In order to describe and quantify the timing of the appearance of a trait, we fit a logistic regression statistical model for each. The utility of logistic regression is that it can model a binary response (in this case presence or absence of a trait) with respect to a continuous predictor variable (fish age or standard length). The inflection point of the logistic regression allows estimation of the characteristic age or standard length at which the majority of individuals transition to having a trait, and the steepness of the fit around the inflection point provides information on variation among individuals in the first appearance of traits. For example, a steep slope will create a nearly stepwise-function that can indicate a high level of synchronization of this transition among individuals, whereas a shallow slope highlights much more variation in the initiation of the trait. We therefore used the fitted logistic model for each trait to find the inflection point as an estimate of the characteristic timing of initiation (or presence) of the trait in the population. Variation in presence of this trait is quantified via the steepness of the curve and presented as the width of the box and whiskers around the inflection point estimate for each trait (see Supplementary Figure S2, e.g., of logistic regressions). Visualizing the logistic regression in this way is imperfect for traits that sometimes will not develop (in particular, some pelvic traits in DER2) — because destructive sampling prevents knowing fated absences for all fish — in which case the appearance of developmental delay and variation might be exaggerated. Because of this, we also present the logistic regressions themselves of the pelvic cartilages and pelvic plates (Supplementary Figure S2), which clearly show relative delay of pelvic traits in DER2 fish that develop them.

3 | RESULTS

We quantified variation in the development of derived skeletal traits that differ among two freshwater populations (DER1 and DER2) and one ancestral marine population (ANC) of threespine stickleback (Figure 1).

3.1 | Ancestral and derived populations are largely congruent in much of post-embryonic skeletogenesis

The developmental sequence and timing of the cartilage and bony elements of the medial fins, dorsal spines, and specific aspects of the pelvic structure are highly congruent in ancestral and derived populations. The overall post-hatching growth rate is tightly correlated with age and is nearly the same in fish from the three compared populations (Supplementary Figure S3).

Caudal, dorsal, and anal fins, primarily used for stabilization and locomotion, follow the same sequence of development and timing of ossification in ancestral and derived populations. First to appear are the hypural cartilages, which are plate-like expansions of the terminal vertebrae that support the caudal fin, followed by the appearance of the radial cartilages of the dorsal and anal fins. Mineralization of the fin rays follows, first in the caudal fin and then in the dorsal and anal fins. Later the cartilages also begin to ossify (Figure 2), starting with the caudal hypurals, followed by the anal and dorsal fin radials, which develop in near unison. This developmental order — caudal fin first followed by both anal and dorsal fins — may be a widespread pattern among teleosts (Bird & Mabee, 2003). The timing of these events is nearly the same in all three populations; the only exception is in the appearance of the cartilage of the caudal hypurals. These cartilages appear slightly earlier in ANC than in DER1 and DER2 (Figure 2).

The three dorsal spines (the "stickles") each comprise a medial serrated spine articulating with a bony support structure. The pelvic structure is a bilateral pair of bony plates, elaborately winged and supporting a ventral pair of spines (Figure 1a). Dorsal and pelvic spines can be erected perpendicular to the body and locked in place to thwart gape-limited predators (Hoogland, Morris, & Tinbergen, 1956; Reimchen, 1983). Together, the processes of the pelvic girdle, the supporting lateral plates, and the dorsal spines' basal plates gird the fish against compression during attack by a predator (Reimchen, 1983).

Many aspects of the development of the dorsal and pelvic spines parallel the development of fin rays, with which they are presumed to share a common evolutionary history as serial homologs (Stiassny & Moore, 1992). The spines are preceded by the appearance of cartilage rods,

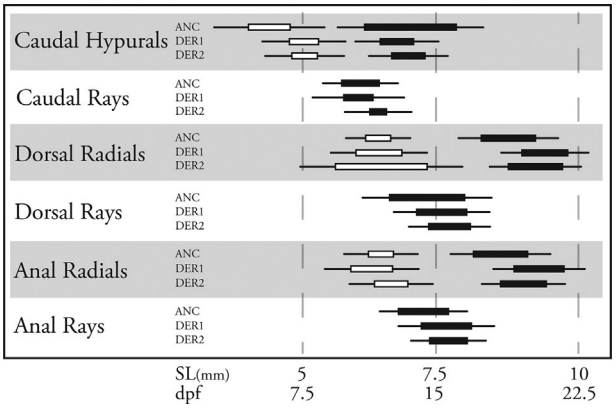

FIGURE 2 Cartilage and bone elements of the medial fins in the two derived populations develop in the same order and initiate at nearly the same time as in the ancestral population. White boxes designate the appearance of cartilage and black boxes designate initiation of ossification

lying beneath the midline in the case of the dorsal spines or just lateral to the ventral midline for each of the pelvic spines (Figures 3a and S4a). The remaining elements are dermal or membrane bone and are not prefigured by cartilage. Mineralization of the spines and a small ray in the pelvic structure follows. Each spine or ray arises first as paired, ossified rods that later converge and fuse at their distal tips (Figures 3b and S4b). At the same time, plates of bone appear, superficial to the mineralizing cartilage rods (Figures 3c and S4c). These plates enlarge, forming the pelvic girdle, ventrally, or the basal plates for each of the dorsal spines along the dorsal midline. Development of the pelvic structure continues with the outgrowth of ascending, anterior, and posterior processes and, just as in the basal plate of the dorsal spines, with the coordinated formation of a foramen at the base of each spine (Figures 3d, S4d, and S4e). The mature foramina are bowl-shaped indentations that articulate with the overlying spine, but in early spine formation they are circular holes through the bone. Finally, sutures are completed between the lateral halves of the anterior (Figure 3e) and posterior processes (Figure 3f) of the pelvic structure (also described in Bell & Harris, 1985).

The developmental sequence and timing of all three dorsal spine elements is conserved among the three populations, though there is discordance in the appearance of the foramina in the supporting structures (Figure 4). Overall the timing of the closure of the circle of each foramen is congruent in ANC and DER1 but relatively delayed in DER2. Similarly, ANC and DER1 concord in the developmental sequence and timing of the pelvic complex, the development of the ascending process, spines, rays, spine foramina, and the suturing of the anterior and posterior processes (Figure 5a). However, this pelvic sequence and timing is largely disrupted in DER2.

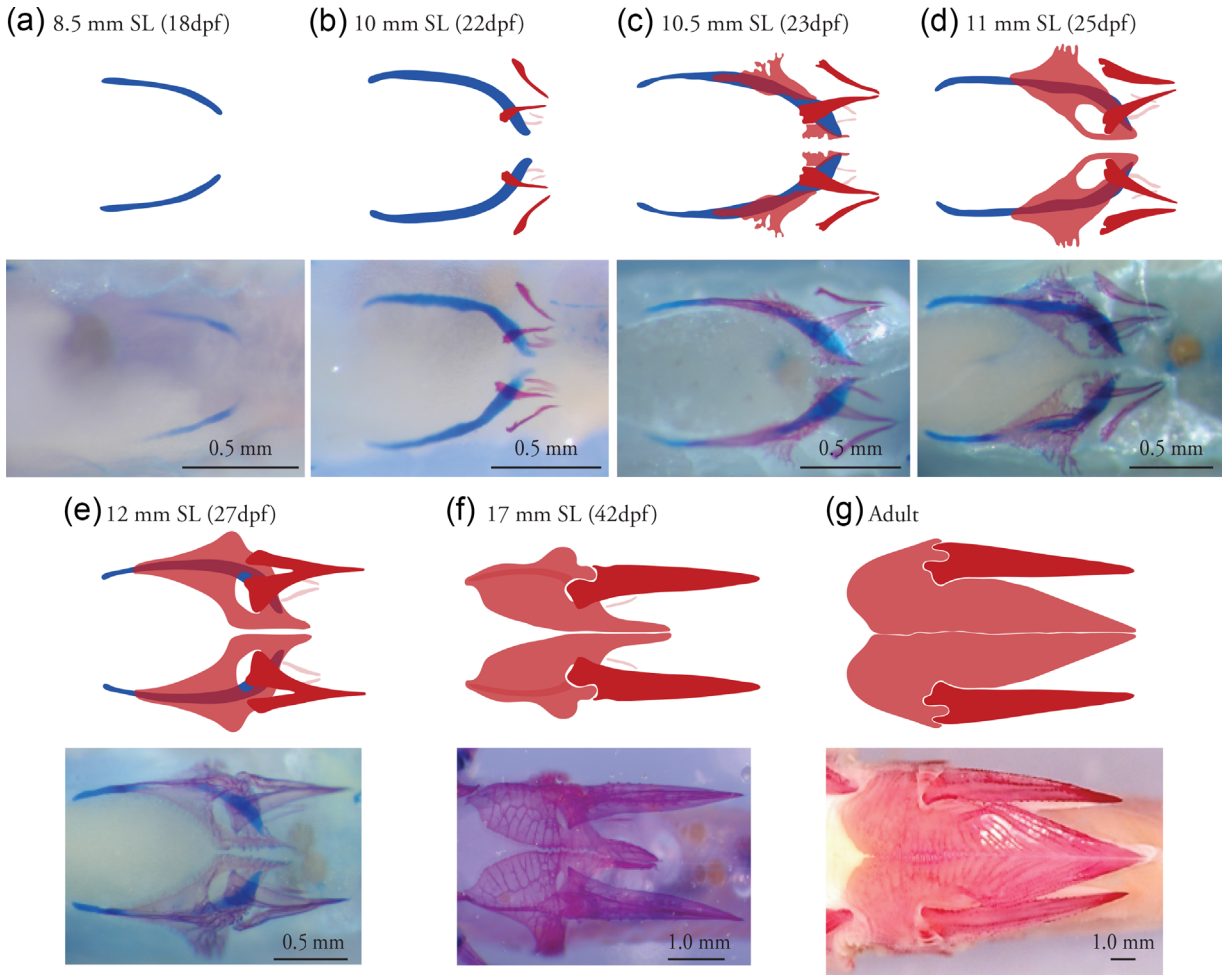

FIGURE 3 Pelvic structure ontogeny in ANC. (a) A cartilage rod forms first. (b) Ossification and outgrowth of pelvic spines and rays. (c) Ossification of the posterior and ascending processes. (d) Ossification of anterior process and continued outgrowth of the posterior and ascending processes and closure of the foramen. (e) Continued outgrowth of all elements. (f) Suturing of the paired posterior processes. (g) Adult pelvic structure. In both the images and illustrations blue labels cartilage and red labels bone

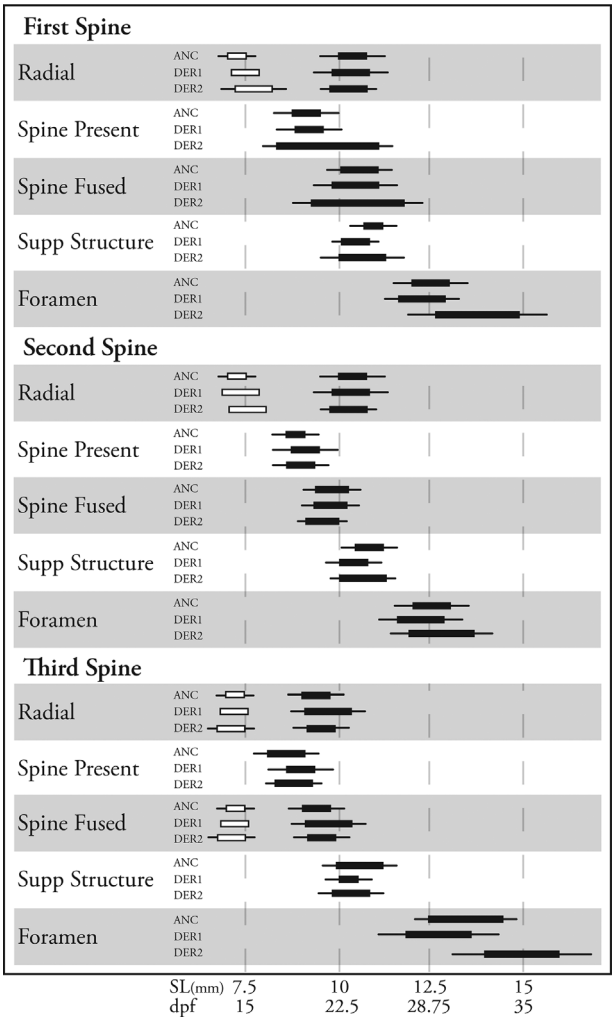

FIGURE 4 Cartilage and bone elements of the three dorsal spines develop in the same order and initiate at nearly the same time in the ancestral and two derived populations. White boxes designate the appearance of cartilage and black boxes designate initiation of ossification

3.2 | Divergent defensive skeletal armor traits occur via altered initial development

Congruence of the developmental chronology of many morphological traits among the three populations, as described above, shows that the clearly documented differences in their adult morphologies are not due to an overall heterochronic shift in development. Rather, there has been modular alteration to particular traits, especially those important for anti-predator defense, as illustrated by the development of the lateral plate armor and the pelvic structure.

Instead of scales that are typical of many other bony fishes, fully armored marine stickleback have a continuous row of dermal bony plates arranged one per body segment along each side of the fish. These "lateral plates" can be classified loosely into different groups (Figure 1a) based on differences in shape, position, and function (Hagen & Gilbertson, 1972; Reimchen, 1983, 2000), though shapes and functions grade into one another. Rostrally, two or three small plates called "anterior plates" are followed by four or five large "supporting plates," which underlap the ascending process of the pelvic structure and the basal plates of the dorsal spines. The remaining are the "posterior plates." A subset of these along the caudal peduncle are called "keel plates" because they bear a prominent horizontal flange. The supporting plates are developmentally the first to ossify (Igarashi, 1965). Mineralizing foci appear first around the lateral line neuromasts (Supplementary Figure S5) and gradually extend dorsally and ventrally (Supplementary Figure S5). Initiation of supporting plates is followed by the ossification of the anterior plates. The keel plates begin mineralizing next, and finally, the rest of the posterior plates fill in bidirectionally from the supporting plates and from the keel plates until each segment is plated (also observed by, Bell, 1981).

The derived lateral plate morphology of the freshwater populations follows delayed ossification of the supporting plates and abolished ossification of posterior plates. In ANC, supporting plates start to appear between 12 and 13 mm SL (27–30 dpf). These plates mineralize slightly later in DER1, but in DER2 they appear over a much later and broader time window, between 16 and 18 mm SL (38–44 dpf) (Figure 5b). No posterior plate ossification was observed (either in these developmental stages or in adults) in the two derived populations, indicating that the developmental change leading to an absence of plates in these segments precedes initiation of mineralization. These observations are consistent with evidence that alleles of *ectodysplasin* (*eda*) are associated with plate loss in stickleback (Colosimo et al., 2005; O'Brown, Summers, Jones, Brady, & Kingsley, 2015), and consistent with the early role of *eda* signaling in formation of vertebrate integumentary appendages, including scales (Kondo et al., 2001; Harris et al., 2008).

Changes in initiation time and in sequence of events appear to have accompanied the evolution of specific pelvic structure morphologies. In ANC and DER1, the pelvic cartilage rod appears between 8 and 9 mm SL (16–19 dpf). By contrast, this component in DER2 is dramatically delayed, until much later between 12 and 14 mm SL (27–33 dpf) (Figures 5a and S2). Ossification of this element starts between 10 and 12 mm SL (22–27 dpf) in the ANC and DER1, and not until 13–17 mm SL (30–41 dpf) in DER2 (Figures 5a and S2). There is a delay of the ossification of the overlying bony pelvic plates in DER2, beginning between 14 and 18 mm SL (33–44 dpf), as compared to mineralizing simultaneously with the cartilage, between 10 and 11 mm SL (22–24 dpf), in ANC and DER1. For all of these traits, the window of initiation is much broader in DER2 with respect to both ANC or DER1 (Figures 5a and S2). However, as stated

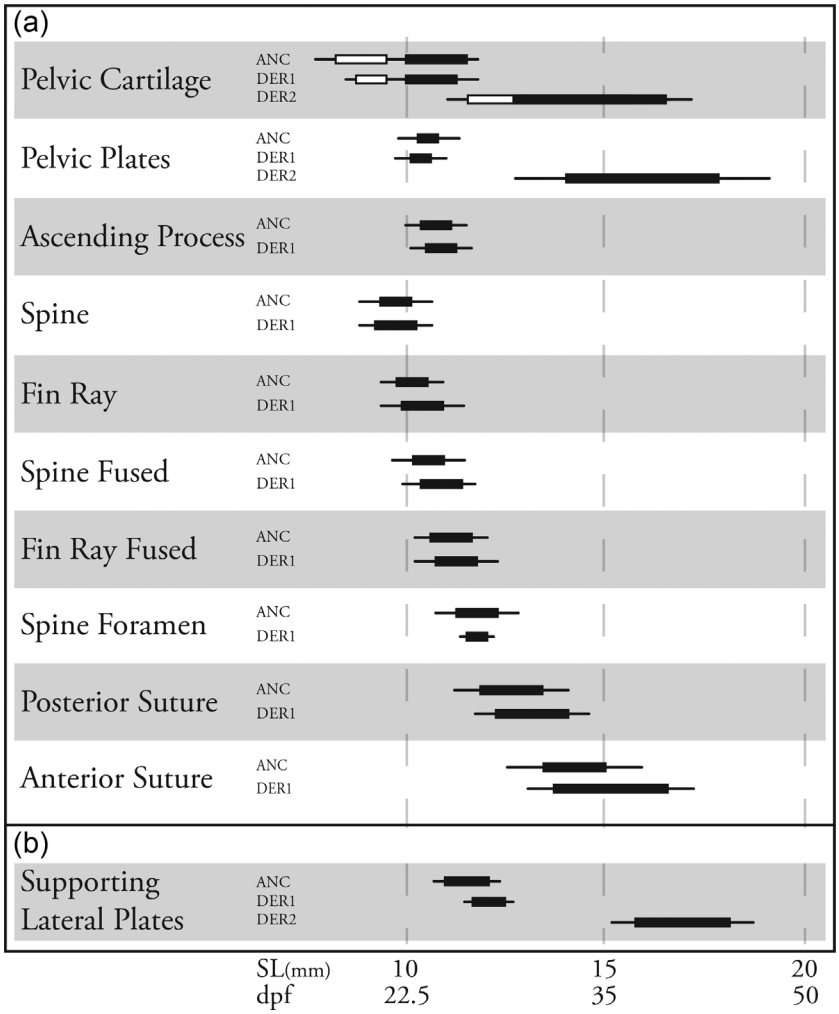

FIGURE 5 Derived populations differ from each other in pelvic and lateral plate development. (a) Developmental delay and variation of the pelvic cartilage and its subsequent ossification, as well as complete failure to initiate some pelvic bones, distinguishes DER2. (b) Despite sharing a similar lateral plate count morphology, the two derived populations initiate lateral plate development at different times. White boxes designate the appearance of cartilage and black boxes designate initiation of ossification

in the methods, destructive sampling could exaggerate the inferred variance, because each sampled individual may or may not have ever developed a given scored pelvic element. We observed that in DER2, anterior and posterior processes (here scored together as pelvic plates) never fully developed, truncated to mere remnants of those seen in ANC and DER1. The ascending processes in DER2 failed to develop at all, as well as the pelvic spines, rays, spine foramina, and the suturing of the posterior and anterior processes.

4 | DISCUSSION

Comparative studies of ontogenetic sequence and timing can be a powerful approach in inferring the developmental-genetic processes that underlie the evolution of adaptive traits. Comparisons of osteogenic sequence and heterochronies among vertebrate taxa have found differences related to important life history traits, and that may underlie evolutionarily divergent aspects of adult skeletal morphology (Weisbecker, Goswami, Wroe, & Sanchez-Villagra, 2008; Wilson et al., 2010; Koyabu et al., 2011; Carril & Tambussi, 2017). Relatively few studies have leveraged a potential point of entry into the mechanisms of vertebrate morphological evolution: variation of the developmental timing within a species. Some existing analyses may be limited by small sample sizes (Wiens, 1989; Mitgutsch, Wimmer, Sanchez-Villagra, Hahnloser, & Schneider, 2011), or the studies are not framed in an adaptive context (Chambers, Leggett, & Brown, 1988; Mabee & Trendler, 1996; Moore & Townsend, 2003). Fish species, in which synchronously developing embryos are often plentiful, can help uncover subtle but potentially adaptive developmental differences among populations (Kawajiri, Kokita, & Yamahira, 2009). Our investigation into variation in developmental timing of adaptive traits that differ among threespine stickleback

populations shows that divergent adult morphologies emerge through altered developmental programs for specific traits in an otherwise highly congruent developmental context. We also show that, of the traits we explored, developmental timing among populations varies the most for defensive armor traits, and that the population with the most divergent morphology also is the most delayed and sports the broadest variation among individuals in ontogenetic timing of defensive traits.

The bony lateral plate armor and its repeated evolutionary loss in derived stickleback populations have a long history of scientific interest (Hagen, 1967; Hagen & Gilbertson, 1972; Hubbs, 1929; Miller & Hubbs, 1969; Reimchen, 1994), with considerable research effort aimed at testing the link between lateral plate variation and ecological correlates, such as types of predation experienced (e.g., Reimchen, 1995) and the availability of dissolved ions in the environment (e.g., Giles, 1983; Marchinko & Schluter, 2007). Other research has focused on discovering the developmental genetic determinants of lateral plate loss (Colosimo et al., 2005). Though the plates are presumed to provide protection against puncturing by predators, another critical role of the supporting plates could be to increase the rigidity of the triangular cross-section formed by erected dorsal and pelvic spines, which can thwart swallowing by vertebrate predators (Hoogland et al., 1956; Reimchen, 1983). The degree to which the lateral plates overlap with the supporting plates of the dorsal spines and the ascending processes of the pelvic structure modulates how effective these girding components are in spreading compression and reducing spine deflection (Reimchen, 1983). There is diminished overlap of the lateral plates with the basal plates of the dorsal spines in Boot Lake stickleback (the source of DER2), which also have a greatly reduced pelvis and ascending processes (Bell & Orti, 1994). It is possible, therefore, that the delay and variation we observed in onset of ossification of the supporting plates in DER2 result from relaxed selection on this developmental program that has followed the breakdown of functional interdependence of ancestral girding components (i.e., the dorsal spines, lateral plates, and pelvic structure) in the absence of piscivorous fish in Boot Lake. The fact that DER1 and DER2 arrive at a roughly parallel compliment of adult lateral plates, but differ markedly in the schedule of their development implicates additional loci — beyond *eda* — that could affect initiation timing and subtler morphological differences such as the smaller plate size and deficit in anterior plates seen in DER2 (Aguirre, Patricia, & Bell, 2004; Colosimo et al., 2004; Cresko et al., 2004; Hagen, 1973; Hermida, Fernández, Amaro, & Miguel, 2002).

The pelvic armor also manifests variation in developmental timing among populations. The stickleback pelvic structure and musculature share homology with the paired teleost pelvic fins, which are more deeply homologous (Stiassny & Moore, 1992) to the hind limbs of tetrapod vertebrates. In QTL mapping analyses, a single genomic locus explained the majority of the phenotype in stickleback with reduced or absent pelvic structures (Cresko et al., 2004; Shapiro et al., 2004). Fine mapping ultimately led to the discovery of deletion haplotypes that ablate the function of an enhancer for pelvic expression of *pitx1*—one of the earliest acting genes described in tetrapod hind limb bud outgrowth (Lanctot, Lamolet, & Drouin, 1997; Szeto et al., 1999)—in several reduced-pelvis stickleback populations (Chan et al., 2010). In DER2, we see a delay in the initial appearance of the pelvic cartilage, if it forms at all, and variation in the timing of the cartilage, of its ossification, and of the expansion of pelvic plates. Surveys of wild-collected stickleback catalogued variation in the superficial components of the pelvic apparatus (Bell, 1987; Bell et al., 1985; Bell & Orti, 1994). Here we show that such differences are predicted by the presence or absence of the pelvic cartilages (Supplementary Figure S6), consistent with a hypothesis that these bony plates, like the basal plates of the dorsal spines, are apolamellar outgrowths from the periostia of the underlying cartilages (Witten & Huysseune, 2007). It is possible that in DER2 stickleback, extant but hypomorphic levels of *pitx1* (Bell, Ellis, & Sirotkin, 2007) or background expression levels of its downstream target *tbx4* (and other downstream regulatory genes) (Duboc & Logan, 2011) permit occasional expression of reduced pelvic traits in some individuals while in others there is a complete failure of hind limb initiation.

The shape and location of the pelvic cartilages we observe by alcian staining are strikingly similar to the *pitx1* expression pattern described by Shapiro et al. (2004). Comparing between our studies, this expression appears to directly precede the appearance of the cartilages and the ossification of the pelvic spines and rays. Interestingly, *pitx1* expression appears strongest where the pelvic spines will emerge, positioned at the posterior ends of the cartilages. In complete-pelvis fish from ANC and DER1, we see that the pelvic spine and ray ossify earlier than either the pelvic cartilages or the overlying plates. Ossified pelvic cartilage and plates sometimes occur in DER2 fish, but they never have pelvic spines or rays. The failure to initiate fin bud outgrowth (Cole et al., 2003) and form a spine is therefore unlikely to be due simply to a truncation of the fin developmental sequence. It is possible that fin bud outgrowth has a higher threshold of *pitx1* expression required to initiate than do the cartilage and pelvic plate anlage (Bell et al., 2007). The variable expressivity of the low-pelvis phenotype we see in DER2 could be due to epistasis such as among identified modifier loci and *pitx2* (Bell et al., 2007; Cresko et al., 2004; Marcil, 2003; Peichel et al., 2001; Shapiro et al., 2004), due to subtle environmental perturbation despite our controlled rearing conditions, or due to stochasticity in expression networks such as might be caused by a near-threshold level of *pitx1* or downstream

targets. That low-pelvis fish still harbor developmental competence to generate components of the pelvic apparatus, such as was observed here and by others (e.g., Bell et al., 2007) and was functionally tested by Chan et al. (2010), suggests a potential for atavism or novel pelvic modification even in lineages with deleted *pitx1* enhancer alleles, such as in Boot Lake (Figure S8 in Chan et al., 2010), which has recently experienced an influx of novel predators (Wund et al., 2015).

In this work, we identified when, during ontogeny, freshwater stickleback diverge from the inferred ancestral program. We found that altered adult phenotypes result from apparently modular developmental changes specific to the divergent traits, which is consistent with genetic mapping studies that identified independent genetic bases of variation in these traits (Colosimo et al., 2004; Cresko et al., 2004; Shapiro et al., 2004), rather than from a global heterochronic shift (Cole et al., 2003). Relaxed selection in one derived population on an integrated girding armor complex that includes the dorsal spines, lateral plates, and pelvic structure has likely created differences between the two derived freshwater populations in degree of variation in developmental timing of these structures. Our findings in post-embryonic skeletal development of stickleback highlight the strength of a "micro-evo-devo" research program in aid of a deeper evolutionary understanding and formulation of new testable hypotheses that help better link underlying genetic variation to evolving phenotypes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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