Canadian Journal of Zoology

Article

Adaptive divergence of lateral plate ultrastructure in threespine stickleback (*Gasterosteus aculeatus*)

Debra L. Wertman and Thomas E. Reimchen

Department of Biology, University of Victoria, PO Box 1700, Victoria, BC V8W 2Y2, Canada

Corresponding author: **Thomas E. Reimchen** (email: reimchen@uvic.ca)

Abstract

The lateral plates of threespine stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) are well studied for their adaptive morphological responses to predators, yet it is unknown whether habitat influences plate ultrastructure. We investigated using scanning electron microscopy the lateral plate ultrastructure (tubercles and ridges) of stickleback ($N = 61$ adult fish) from nine Haida Gwaii (coastal British Columbia, Canada) wild-type populations, two experimental transplants, and two lab-reared cohorts reared from source populations. Tubercle density, but not ridge density, differed significantly across habitat types and populations. Among wild-type fish, tubercle densities were greatest in dystrophic habitats containing predatory fish, and lowest in weakly dystrophic systems featuring bird–invertebrate predation and marine populations with diverse predatory fish. No differences in tubercle density were detected between source and transplant populations, despite major habitat shifts. Lab-reared fish exhibited significantly lower tubercle densities than their source populations (less than one generation). Tubercle density differences across habitat types may reflect adaptation to divergent predation regimes, with tooth-bearing predators selecting for denser tubercles that disperse point forces. Conservation of ridge density across populations suggests an essential function in dispersing forces applied to dorsal spines during predator manipulation. Lateral plate ultrastructure in threespine stickleback thus results from both heritable effects and developmental plasticity.

Key words: *Gasterosteus aculeatus*, threespine stickleback, dermal armour, tubercle, SEM, adaptive radiation, Haida Gwaii

Résumé

Si les plaques latérales des épinoches à trois épines (*Gasterosteus aculeatus* Linnaeus, 1758) ont été bien étudiées pour leurs réactions morphologiques d'adaptation aux prédateurs, une éventuelle influence de l'habitat sur l'ultrastructure de ces plaques n'a pas été établie. Nous étudions, à l'aide de la microscopie électronique à balayage, l'ultrastructure des plaques latérales (tubercules et crêtes) d'épinoches ($N = 61$ poissons adultes) de neuf populations sauvages de l'archipel Haida Gwaii (Colombie-Britannique, Canada), deux populations transplantées expérimentales et deux cohortes élevées en laboratoire issues de populations sources. La densité de tubercules, mais non celle de crêtes, varie significativement d'un type d'habitat et d'une population à l'autre. Parmi les poissons de type sauvage, les densités de tubercules sont les plus grandes dans des habitats dystrophiques contenant des poissons prédateurs et les plus faibles dans des systèmes peu dystrophiques caractérisés par la prédation d'oiseaux et d'invertébrés et des populations marines comptant une diversité de poissons prédateurs. Aucune différence de la densité de tubercules n'est décelée entre les populations sources et transplantées, malgré d'importants changements d'habitats. Les poissons élevés en laboratoire présentent des densités de tubercules significativement inférieures à celles de leurs populations sources (moins d'une génération). Les différences de densité de tubercules entre types d'habitat pourraient refléter l'adaptation à des régimes de prédation différents, les prédateurs dentés sélectionnant des individus à tubercules plus denses qui dispersent les forces ponctuelles. La conservation de la densité de crêtes entre populations indiquerait une fonction essentielle de dispersion des forces appliquées aux épines dorsales durant la manipulation par les prédateurs. L'ultrastructure des plaques latérales chez les épinoches à trois épines est donc le résultat à la fois d'effets héréditaires et de la plasticité développementale. [Traduit par la Rédaction]

Mots-clés : *Gasterosteus aculeatus*, épinoches à trois épines, armure dermique, tubercule, MEB, rayonnement adaptatif, Haida Gwaii

Introduction

Dermal armour in animals provides protection from environmental threats including predation attempts, functioning to minimize damage to underlying soft tissue that may result in injury, infection, or death (Romer 1933; Vermeij 1993; Bruet et al. 2008). Convergence of dermal armour

towards strong but flexible structures composed of rigid units, often with complex microstructure connected by collagen fibres and muscle, has occurred repeatedly among diverse vertebrate taxa including fish (Bruet et al. 2008; Song et al. 2010, 2011; Yang et al. 2012, 2013; Ebenstein et al. 2015). Production of dermal armour can also result in added mass (Myhre and Klepaker 2009), reduced flexibility (Bruet et al. 2008; Leinonen et al. 2011), and increased metabolic cost of biomineralization (Mann 2001; Song et al. 2010, 2011; Loewen et al. 2016). Additionally, dermal armour might cloak sensory receptors such as the lateral line in fish (Webb and Shirey 2003; Song et al. 2010; Wark and Peichel 2010). Evolutionary trade-offs therefore exist between potential fitness costs and benefits of dermal armour (Bruet et al. 2008; Song et al. 2010; Leinonen et al. 2011; Yang et al. 2013), and it is unknown how these trade-offs may extend to the ultrastructure of bony armour in fishes.

Stickleback (Gasterosteidae) are a model taxon for evolutionary investigation (Bell and Foster 1994). One widely studied trait of the threespine stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) is the bony lateral plates, which vary widely in number among individual fish from freshwater populations (reviewed in Wootton 1976). Threespine stickleback lateral plates have two primary functions, buttressing of the dorsal and pelvic spines in the mid-trunk to defend against gape-limited predators and providing epidermal protection from puncturing predators (Reimchen 1983, 1992). Threespine stickleback underwent a well-documented adaptive radiation in response to colonization of isolated freshwater systems following glacial retreat ~10 000 years ago in the Haida Gwaii archipelago, located approximately 100 km off the west coast of Canada (Reimchen et al. 2013; Marques et al. 2022). Among these populations, many of the morphological differences in specific traits, such as lateral plate size and number, are functionally associated with the ecological conditions of their habitats including water clarity and predation regime.

In the present study we examined the ultrastructure of lateral plates of 11 ecologically divergent populations of threespine stickleback within the Haida Gwaii archipelago, including 2 marine systems dominated by fish predation, 7 strongly or weakly dystrophic lakes, ponds, and streams with either avian (bird–invertebrate) or fish (fish–bird, bird–fish)-dominated predation regimes, and 2 experimental transplant populations. We additionally analyzed fish from two lab-reared cohorts originating from one wild-type (Mayer Lake) and one transplant (Mayer Pond) population. We chose to assess plates at the sixth and seventh positions (henceforth referred to as LP6 and LP7) due to their biomechanical importance in dispersing major forces transmitted from the basal plate of the second dorsal spine and ascending process of the pelvis during predator manipulation (Reimchen 1983). Marine threespine stickleback possess 30 to 35 lateral plates that extend to the full length of the trunk (Wootton 1976). Freshwater stickleback, which have lost most of the posterior plates and retain up to seven to eight anterior plates, are typically found in systems with tooth-bearing predatory fish that puncture their prey, while populations with fewer plates are found in habitats lacking puncturing predators but containing avian piscivores, which are compression predators, as well as macroinvertebrate-grappling predators (Reimchen 1994). We predicted a distinction in plate ultrastructure between wild-type populations found in marine or dystrophic freshwaters dominated by puncturing predators (fish) versus weakly dystrophic freshwater systems dominated by avian (compression) predators, although we did not have a priori expectations of how these populations would differ.

The two experimental transplant populations, founded by fish transferred from two large source lakes into ponds lacking stickleback within the last three decades, are useful in our study for potential detection of phenotypic plasticity and rapid selection, as the habitats where the fish were transplanted are "ecologically opposite" from each of the source populations. Differences between source and transplant habitats include a shift from dystrophic (blackwater) large lakes with low pH to eutrophic ponds with higher pH and conductivity, from a limnetic to a benthic trophic niche, and from a predation landscape dominated by predatory fish and birds to one dominated by macroinvertebrates and birds. Lab-reared fish, collected as eggs from two source populations (one wild-type and one transplant) and reared in the absence of predators and predator cues, were examined to determine whether plate ultrastructure was conserved in these artificial rearing conditions within a single generation. We expected shifts in plate ultrastructure between source populations and respective transplant and lab-reared fish, based upon previous assessments of morphological data from the Mayer Lake experimental system that implicate influences of both selection and plasticity on bony armour expression (Leaver and Reimchen 2012).

Materials and methods

Threespine stickleback samples ($N = 61$) collected during previous studies in the Haida Gwaii archipelago were selected from 11 populations originating from marine (Masset Inlet, Inskip Lagoon) and freshwater (lake, pond, stream) systems of divergent water clarity, water conductivity, and predation regimes, and from two lab-reared cohorts (Table 1). The fish, bird, and invertebrate species comprising the predator assemblages in these freshwater systems are described in Reimchen (1994). Boulton Lake, Richter Lake, and the adjacent Middle Pond contain threespine stickleback populations that lack exposure to predatory fish and exhibit the greatest relative reduction of bony armour (Reimchen et al. 2013). Boulton Lake fish are also deeply split phylogenetically from the other freshwater populations (Deagle et al. 2013), and thus comparisons between Boulton Lake and other wild-type populations should reveal the extent of morphological divergence in wild-type populations. Other comparisons of interest included adjacent lake–stream (Mayer Lake–Gold Creek, Drizzle Lake–Drizzle Outlet; Reimchen et al. 1985; Deagle et al. 2012) and lake–pond (Richter Lake–Middle Pond) populations, source–transplant populations (Mayer Lake–Mayer Pond, Drizzle Lake–Drizzle Pond), and source–lab-reared populations (Mayer Lake–Mayer Lake Lab, Mayer Pond–Mayer Pond Lab), which are each paired by ancestry (Deagle et al. 2013). Mayer Pond and Drizzle Pond contain experimental

Table 1. Summary of biophysical variables and dominant predation regimes (fish–bird, bird–invertebrate) of all 13 threespine stickleback (*Gasterosteus aculeatus*) populations/cohorts from the Haida Gwaii archipelago surveyed in this study.

Location	Type	Area (ha)	Depth (m)	Water clarity (% transmission at 400 nm)	System productivity	Conductivity (mS/cm)	Dominant predation	Number of fish
Masset Inlet	Marine	—	—	99	—	13 400	Fish–bird	4
Inskip Lagoon	Marine	—	—	99	—	13 400	Fish–bird	2
Boulton Lake	Lake	15	4	78.1	Weakly dystrophic	~45	Bird–invertebrate	3
Middle Pond	Pond	1	2	55.1	Weakly dystrophic	~160	Bird–invertebrate	3
Richter Lake	Lake	12	5	51.9	Weakly dystrophic	~160	Bird–invertebrate	4
Drizzle Outlet	Stream	—	0.5	67.0	Dystrophic	45	Fish	3
Drizzle Lake	Lake	97	16	67.0	Dystrophic	~50	Bird–fish	9
Drizzle Pond	Pond	<1	1	76	Eutrophic	~80	Bird–invertebrate	7
Gold Creek	Stream	—	<2	50.2	Dystrophic	~100	Fish–bird	3
Mayer Lake	Lake	490	20	57.1	Dystrophic	~90	Fish–bird	8
Mayer Pond	Pond	0.25	1	~75	Eutrophic	~160	Bird–invertebrate	7
Mayer Lake Lab	Lab-reared	—	—	99	—	60	None	5
Mayer Pond Lab	Lab-reared	—	—	99	—	60	None	3

Note: Conductivity data can vary ±20% among years for freshwater habitats. Sixty-one fish were evaluated, with the number of fish per population/cohort indicated. No entry (—) indicates missing or inapplicable data. Conductivity is positively associated with calcium availability and inversely related to pH. For location maps see Spoljaric and Reimchen (2008).

transplant populations that were founded by fish from their source locations, Mayer Lake and Drizzle Lake, in 1992 (summarized in Leaver and Reimchen 2012) and 1997 (T.E. Reimchen, unpublished data), respectively. Lab-reared fish were obtained as egg clutches from Mayer Lake and Mayer Pond in 2007 and raised to maturity at the University of Victoria Aquatic Facility (Leaver and Reimchen 2012). Fish were euthanized using tricaine methanesulfonate (MS222) in compliance with University of Victoria Aquatic Unit Standard Operating Procedure OA2003, collected in buffered formalin (10%) or alcohol (70% isopropyl or 95% ethanol) and later transferred to fresh 95% ethanol for long-term storage.

Lateral plates LP6 and LP7 from the left and right sides of 61 adult fish (53–84 mm standard length) were removed by dissection and isolated from one another. Samples were then cleared of tissue by submerging in a 0.35% sodium hypochlorite (bleach) solution, followed by repeated rinsing and soaking in water for 10 min. Under a dissecting microscope, the plates were freed of any remaining tissue and allowed to air dry. Plates were adhered to aluminum scanning electron microscope (SEM) specimen mount stubs using colloidal silver paste and dried for 48 h. Samples were gold-coated in three 1 min cycles using the Edwards Sputter Coater S150B and then visualized under a Hitachi S-3500N Scanning Electron Microscope. High-resolution scans ranging from 40× to 80× magnification were captured for analysis.

Lateral plate tubercle and ridge densities were quantified from SEM images using ImageJ 1.46r (Rasband 2013). Standardized measurements are schematically illustrated in Fig. 1. The number of ridges passing through each 0.5 mm transect and the number of tubercles present in each 0.5 mm^2 quadrat were counted; a feature was included if it fell >50% inside the quadrat or transect line. Ridges were defined as raised regions of mineralized tissue that were generally seen interconnecting tubercles (Fig. 1) and were considered to be present if a transect fell directly over a tubercle that was connected by ridging on either side. Tubercle and ridge density measurements were averaged across transects (ridges only, three to four transects per plate), plates (LP6, LP7), and sides (L, R) for each fish. Tubercle and ridge density fish means were rounded to the nearest integer, generating count-based response data for statistical analysis. For the purpose of visualizing raw population-level data, tubercle and ridge density means and standard errors were calculated across fish for each population.

A Spearman's rank correlation was performed to evaluate the relationship of tubercle and non-normally distributed ridge density counts across all populations and cohorts. Based upon this test result we proceeded to analyze the two response variables separately. We constructed a total of eight Poisson regressions to investigate the effects of habitat type, genetic relatedness, experimental transplant, and lab-rearing on tubercle and ridge density in our sample set (Table 2). Two models were generated to test the effect of habitat type (marine, dystrophic freshwater, and weakly dystrophic freshwater) on tubercle and ridge density in wild-type fish, and two additional analyses performed to target specific tubercle and ridge density comparisons between genetically related wild-type populations (population $N = 9$). Two models were devel-

Fig. 1. Schematic of dissection, and measurements (tubercle and ridge density) performed on SEM images, of threespine stickleback (*Gasterosteus aculeatus*) lateral plates (Mayer Lake example, left side shown). Transects and quadrats were size-standardized, and measurements performed, in ImageJ (Rasband 2013). Asterisks (∗) mark guiding lines (length based upon plate width) for transect placement, extending vertically from the central neuromast pore; T2, T3, and T4 are 0.5 mm horizontal transects placed for ridge density quantification; Q is a 0.5 mm^2 quadrat for tubercle density quantification placed dorsally to the most dorsal pore, with the midpoint of the ventral side of the quadrat aligned with the posterior edge of the dorsal pore. In case of insufficient dorsal plate area, quadrats and transects were positioned as close as possible to the initial target region. Ridges were defined as raised regions of mineralized tissue found interconnecting tubercles; in this example, six ridges pass through T2, five through T3, and four through T4. Lateral plates LP6 and LP7 were examined from the left and right sides of fish collected from 13 freshwater, marine, and lab-reared populations throughout the Haida Gwaii archipelago (coastal British Columbia, Canada).

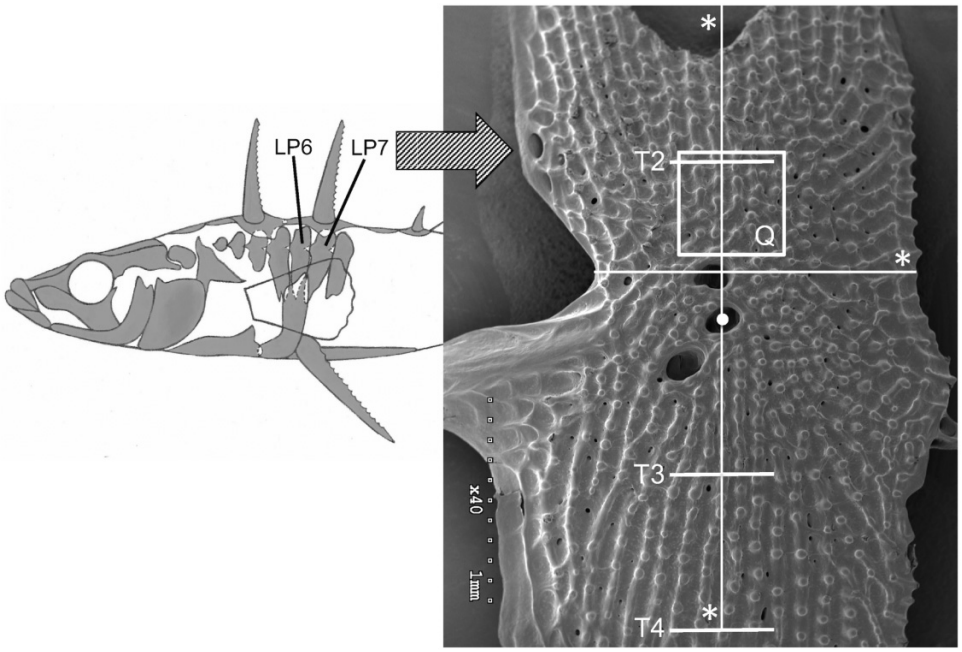


Table 2. Summary of eight Poisson regressions (log-link function), selected using a backwards stepwise method, implemented for analysis of threespine stickleback (*Gasterosteus aculeatus*) lateral plate ultrastructural features in relation to habitat type (dystrophic, weakly dystrophic, marine) or population and fish standard length.

Model subject	Habitat type/ population *N*	Response variable	Fixed-factor explanatory variable(s)
Habitat type (wild-type)	3	Tubercle density	Habitat type, standard length
Habitat type (wild-type)	3	Ridge density	Habitat type
Wild-type	9	Tubercle density	Population, standard length
Wild-type	9	Ridge density	Population
Transplant	4	Tubercle density	Population, standard length, population × standard length
Transplant	4	Ridge density	Population
Lab-reared	4	Tubercle density	Population
Lab-reared	4	Ridge density	Population

Note: Models were developed to assess the effects of habitat type, genetic relatedness, experimental transplant, and lab-rearing on lateral plate tubercle and ridge density. Mature fish ($N = 61$) were collected from 13 freshwater and marine wild-type ($N = 9$), experimental transplant ($N = 2$), and lab-reared populations ($N = 2$) throughout the Haida Gwaii archipelago (coastal British Columbia, Canada). SEM images of plates LP6 and LP7 from the left and right sides of the fish were analyzed for tubercle (per 0.5 mm^2 quadrat) and ridge (per 0.5 mm transect, mean of three to four transects per plate) densities using ImageJ 1.46r (Rasband 2013), with values averaged to the nearest integer across individual fish for analysis.

oped to investigate the effects of experimental transplantation on tubercle and ridge density relative to wild-type source populations (population $N = 4$). Finally, two models were constructed to isolate any phenotypically plastic effects on tubercle and ridge density in lab-reared fish relative to their source populations (population/cohort $N = 4$). Fish standard length, a measure of body size, was incorporated into our models as a fixed factor covariate; in the case of one missing Masset Inlet

standard length measurement, the population average was assigned to the fish as its standard length.

Final models were selected using a backwards stepwise method, beginning with complete models containing habitat type/population, standard length, and habitat type/population × standard length as fixed effects. Generalized linear Poisson regression (log-link function) was implemented for each full model to accommodate count-based response data (tubercle and ridge density per unit area/transect, respectively). Poisson regression was used conservatively to model underdispersed data, detected in all ridge density models, and wild-type population-level and transplant tubercle density analyses. If terms or interactions were not significant in a model, then these factors were sequentially dropped from the analysis until only the variable of interest (habitat type/population) remained (Table 2).

Analyses were performed in RStudio (RStudio Team 2021; R Core Team 2021), model dispersion was assessed using DHARMa (Hartig 2019), and pairwise post hoc comparisons between populations in the Poisson regressions were produced using the emmeans package (Lenth 2019). Plots were generated using ggplot2 (Wickham 2016), implementing the visreg package (Breheny and Burchett 2017) to visualize the models.

Results

Overall, the ultrastructure of the plates had linear arrays of tubercles interconnected by ridges of variable height radiating dorsoventrally from the central neuromast pores (Fig. 2). Tubercle and ridge densities ($N = 61$ fish) were significantly but not perfectly correlated across all populations and cohorts (Spearman's correlation coefficient $\rho = 0.54$, $P < 0.0001$). Tubercle densities of wild-type fish differed significantly across habitat types ($P < 0.0001$, deviance = 25.47 on 2 df) (Fig. 3A) and were negatively associated with standard length ($P < 0.01$, deviance = 10.44 on 1 df). Tubercle density, similarly affected by standard length ($P < 0.01$, deviance = 7.18 on 1 df), also varied significantly across wild-type populations ($P < 0.0001$, deviance = 41.57 on 8 df). In contrast, ridge densities of wild-type fish were consistent across habitat types ($P = 0.95$, deviance = 0.10 on 2 df) and populations ($P = 1.00$, deviance = 0.80 on 8 df), with no influence of standard length on either model. Pairwise post hoc comparisons revealed significantly higher tubercle densities in fish from dystrophic freshwater habitats than those from both marine ($P < 0.0001$) and weakly dystrophic freshwater ($P < 0.001$) habitats, although tubercle densities of fish from marine and weakly dystrophic freshwater habitats did not differ from one another ($P = 0.28$) (Fig. 3A). At the wild-type population-level, no differences in tubercle density were detected between lake–stream pairs (Mayer Lake–Gold Creek ($P = 1.00$) and Drizzle Lake–Drizzle Outlet ($P = 1.00$)), nor between Richter Lake and neighbouring Middle Pond ($P = 0.97$). Population-level pairwise comparisons for tubercle density revealed divergence between Boulton Lake and two dystrophic habitat populations (Mayer Lake ($P < 0.01$) and Gold Creek ($P < 0.05$)).

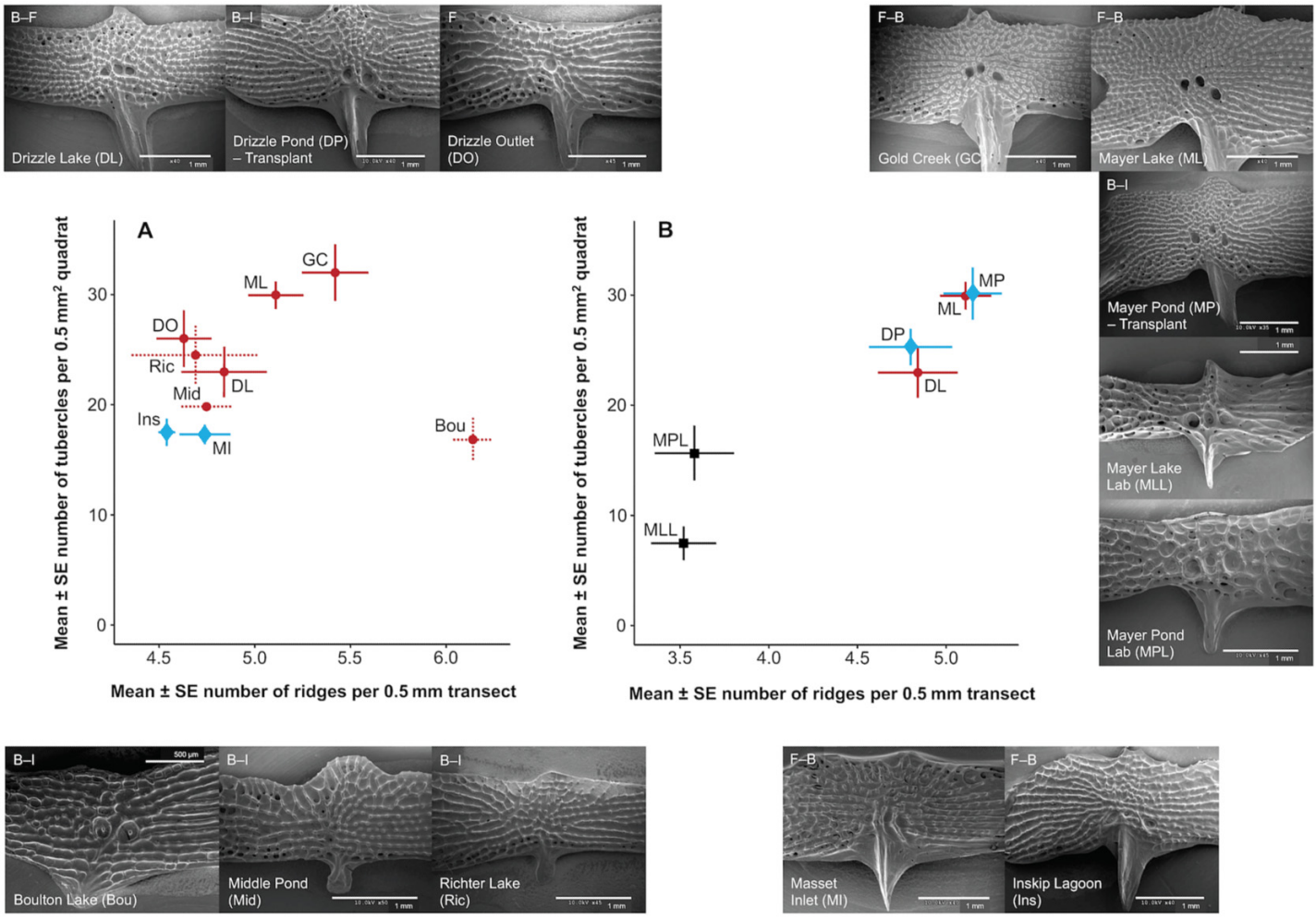
Tubercle density varied significantly among source and experimental transplant populations ($P < 0.05$, deviance = 11.31 on 3 df) and was negatively affected by standard length ($P < 0.05$, deviance = 4.42 on 1 df; population × standard length: $P < 0.05$, deviance = 10.95 on 3 df); however, no differences in tubercle density were detected between source and experimental transplant population pairs (Mayer Lake–Mayer Pond ($P = 0.50$), Drizzle Lake–Drizzle Pond ($P = 0.99$)) (Fig. 3B). Significant differences in tubercle density were detected among source and lab-reared fish ($P < 0.0001$, deviance = 107.56 on 3 df) with no effect of standard length. Pairwise comparisons revealed significant reductions in tubercle density between source populations and lab-reared fish (Mayer Lake–Mayer Lake Lab ($P < 0.0001$), Mayer Pond–Mayer Pond Lab ($P < 0.001$)) (Fig. 3C). Ridge density did not vary between source and experimental transplant populations ($P = 0.99$, deviance = 0.35 on 3 df), nor between source populations and lab-reared cohorts ($P = 0.40$, deviance = 2.92 on 3 df), with no influence of standard length on either model.

Discussion

The results of this study implicate both genetic heritability and phenotypic plasticity in the adaptive expression of lateral plate ultrastructure in Haida Gwaii threespine stickleback. Our characterization of tubercles and ridges are similar with previous descriptions of marine threespine stickleback from Alaska and northern Europe (Song et al. 2010; Lees et al. 2012) suggesting ancestrally conserved structures that, according to our results, are nonetheless subject to differentiation. Tubercle density in wild-type fish differed across marine, dystrophic, and weakly dystrophic freshwater habitat types that consist of stickleback populations that are subject to highly divergent biotic (predator assemblage) and abiotic (for example, water clarity and conductivity) influences, leading us to infer that this variation is likely adaptive. Tubercle density was consistent between lake–stream and lake–pond populations paired by ancestry, and between source and respective experimental transplant populations, indicating strong genetic influence on this trait. Boulton Lake fish tubercle densities differed significantly from Mayer Lake and Gold Creek fish, perhaps resulting from the combination of adaptive divergence to habitat type and phylogenetic distance. Lab-reared fish exhibited highly reduced tubercle densities relative to their respective source populations, revealing that lateral plate ultrastructure is also susceptible to plasticity-induced shifts within a single generation.

Ridge density was highly conserved across habitat types and populations/cohorts, likely indicative of a consistent function in lateral plate spine-buttressing during predator manipulation. The dorsoventral alignment threespine stickleback lateral plate ridges and associated tubercles radiating from the central neuromast pores (described in Planidin and Reimchen 2019), also observed in specimens from Alaska (Song et al. 2010) and northern Europe (Lees et al. 2012), are probably biomechanically important during predator manipulation as force is applied to the erect spines and transferred to the dorsal edge of the lateral plate (Reimchen 1983; Bruet

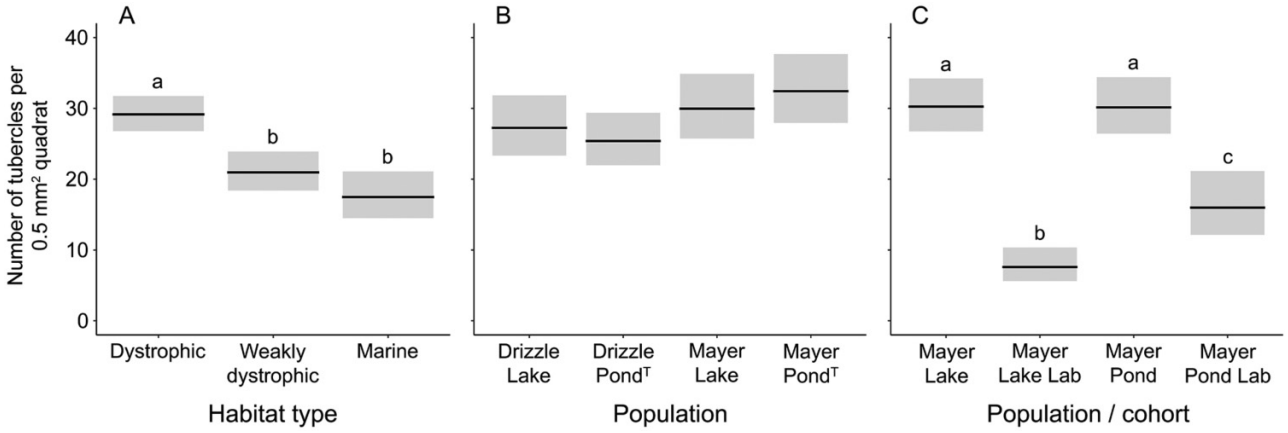
Fig. 2. Scatterplot of population mean ± SE number of tubercles per 0.5 mm^2 quadrat versus mean ± SE number of ridges per 0.5 mm transect for lateral plates from (A) nine wild-type (marine and freshwater) populations, and (B) two experimental transplant populations and their wild-type source populations, and two lab-reared populations/cohorts (and their source populations) of threespine stickleback (*Gasterosteus aculeatus*) from the Haida Gwaii archipelago (coastal British Columbia, Canada). (A) Red circles and solid lines represent dystrophic habitats, red circles and dashed lines indicate weakly dystrophic habitats, and blue diamonds and solid lines represent marine habitats. (B) Red circles and solid lines represent dystrophic source habitats, blue diamonds and solid lines indicate eutrophic transplant habitats, and black squares and solid lines represent lab-reared cohorts. Example lateral plate SEM images for each population/cohort are shown, oriented dorsoventrally left to right; F–B corresponds to (=) populations from habitats dominated by fish–bird predation, B–F = bird–fish predation, F = fish predation, and B–I = bird–invertebrate predation. SEM images of plates LP6 and LP7, cleared of tissue, from the left and right sides of mature fish (N = 61) were analyzed for tubercle and ridge density using ImageJ 1.46r (Rasband 2013). [Colour online.]



et al. 2008). An essential biomechanical function of ossified ridges on the spine-buttressing lateral plates against both gape-limited (fish) and compression (avian) predators may explain the consistency in ridge density across samples.

We suggest that the observed differences in tubercle density among wild-type stickleback largely reflect the divergent predator assemblages found in their respective habitats, paralleling phenotypic trends previously observed in this species (Reimchen 1994; Reimchen et al. 2013). Bony armour (Vermeij 1993; Bruet et al. 2008; Yang et al. 2013), including lateral plates with robust ultrastructure on threespine stickleback (Reimchen 1992, 2000; Song et al. 2010), should provide essential protection against puncturing predators. Dystrophic systems (Mayer Lake, Gold Creek, Drizzle Lake, and Drizzle Outlet) feature salmonid puncturing predators in fish–bird or bird–fish predation regimes (Reimchen 1994), and the lateral plates of the stickleback originating from these habitats display the greatest tubercle densities of all wild-type fish. Densely packed tubercles may protect against puncture by (*i*) maximizing plate thickness while minimizing armour weight (Bruet et al. 2008; Myhre and Klepaker 2009; Song et al. 2010, 2011), (*ii*) maintaining distance between a predator's tooth and the infrastructure of the plate (Song et al. 2010; Ebenstein et al. 2015), and perhaps most importantly, (*iii*) by dispersing point forces exerted during puncturing attacks by toothed predators (Song et al. 2010, 2011; Yang et al. 2012,

Fig. 3. Visualization of the contributions of habitat type (A) and population (B, C) to Poisson regressions (log-link function; including fish standard length as a fixed covariate) describing Haida Gwaii threespine stickleback (*Gasterosteus aculeatus*) lateral plate tubercle density across (A) habitat types ($N = 3$, comprising nine wild-type populations) ($P < 0.0001$, deviance = 25.47 on 2 df; standard length: $P < 0.01$, deviance = 10.44 on 1 df). (B) Source and experimental transplant (indicated by a superscript T) populations ($N = 4$) (population: $P < 0.05$, deviance = 11.31 on 3 df; standard length: $P < 0.05$, deviance = 4.42 on 1 df; population × standard length: $P < 0.05$, deviance = 10.95 on 3 df), and (C) source populations and lab-reared cohorts ($N = 4$) (population: $P < 0.0001$, deviance = 107.56 on 3 df). SEM images of plates LP6 and LP7 from the left and right sides of mature fish were assessed for number of tubercles per 0.5 mm^2 quadrat using ImageJ 1.46r (Rasband 2013). Tubercle density counts were averaged to the nearest integer for each fish. Letter pairs (a–b–c) highlight significant pairwise differences in tubercle density between habitat types (minimum $P < 0.001$), and source and lab-reared populations (minimum $P < 0.01$). No pairwise differences in tubercle density were observed between source and experimental transplant populations. Horizontal lines represent model estimates surrounded by 95% confidence bands.



2013; Ebenstein et al. 2015). Stickleback from small, weakly dystrophic systems (Boulton Lake, Middle Pond, and Richter Lake) characterized by bird–invertebrate predation exhibit low tubercle densities consistent with the lack of puncturing predators under this hypothesis. The retention of great tubercle densities in many wild-type (for example, Gold Creek) fish initially preserved in buffered formalin reveals that preservation method did not affect the mineralization of bony ultrastructure.

Marine stickleback (Inskip Lagoon and Masset Inlet), which are fully plated, display low tubercle densities despite the high levels of piscivorous predation characteristic of oceanic waters. There could be multiple processes contributing to the low tubercle densities in fully plated marine stickleback, including altered flow regime in the boundary layer (Aleyev 1977; Fish and Lauder 2006; Song et al. 2010), maintenance of trunk flexibility (Bruet et al. 2008; Leinonen et al. 2011; Yang et al. 2013), and greater diversity and relative increased size of predatory fish that may exert different or fewer point forces on stickleback lateral plates. Energetic trade-offs in allocation for bone construction could occur (Bourgeois et al. 1994), in that it may be less important for fully armoured marine stickleback to produce robust tubercles for defense against puncturing predators and energy better directed at mineralizing the essential underlying structure of numerous plates (Mann 2001; Leinonen et al. 2011; Loewen et al. 2016). Larger stickleback tended to have lower tubercle densities per unit area than smaller fish, a trend we observed in both wild-type and experimental transplant analyses, revealing that ultrastructure does not display allometric growth. It is possible that decreased tubercle density in larger fish results from increased protection against puncture afforded by proportionally more robust plates, or from body-size-associated differences in flow regime (Aleyev 1977; Fish and Lauder 2006; Song et al. 2010) or trunk flexibility (Bruet et al. 2008; Leinonen et al. 2011; Yang et al. 2013).

Mayer Pond and Drizzle Pond transplant populations demonstrated no shifts in tubercle density from their Mayer Lake and Drizzle Lake source populations, after 17 years (approximately eight generations) and 14 years (approximately seven generations), respectively, despite major biophysical differences in habitat conditions. These experimental ponds are both small, eutrophic, subject to bird–invertebrate predation, and were selected to serve as ecological opposites of the large, dystrophic fish–bird predation dominated source lakes. Previous work has shown that phenotypic (Leaver and Reimchen 2012) and genotypic (Marques et al. 2018) shifts occurred in an ecologically predictable direction from Mayer Lake to Mayer Pond. Our observations suggest that such a change has not occurred in the lateral plate ultrastructure of fish from transplant populations, implicating a high degree of heritability from the source populations. Alternatively, because the transplant ponds have elevated conductivity relative to the source populations, higher calcium availability (Giles 1983; Bourgeois et al. 1994; Loewen et al. 2016) may facilitate expression of the tubercles and counteract any major effects of selection for tubercle reduction.

In spite of shared ancestry, lab-raised Mayer Lake and Mayer Pond samples reveal dramatic shifts from their source populations to an exceptionally reduced tubercle density

phenotype. This result parallels previous documentation of plastic expression of dorsal spine and gill raker lengths in these lab-reared cohorts (Leaver and Reimchen 2012). Individuals were collected as fertilized eggs from their respective source populations, hatched and reared in the laboratory, and thus the observed shifts in tubercle densities mechanistically implicate intragenerational, plastic responses to environmental stimuli. Despite the relatively low conductivity of the water containing the lab-raised fish relative to the source populations (60 versus 90 mS/cm (Mayer Lake) and ~160 mS/cm (Mayer Pond)) calcium levels are an order of magnitude higher in lab waters (M. Gordon, personal communication, December 2021), suggesting that the low tubercle density in lab-reared fish cannot be due to deficiencies in calcium that is essential for bone construction, as suggested by some previous studies (Giles 1983; Bourgeois et al. 1994; Loewen et al. 2016). Plastic expression of stickleback phenotypes, including morphology (body shape and individual traits) and growth rate, has been experimentally demonstrated in response to predator cues (Frommen et al. 2011; Ab Ghani et al. 2016), as well as to salinity (Mazzarella et al. 2015) and diet shifts (Day et al. 1994). We conclude, based on the cumulative trends from the wild-type, transplant, and lab-reared populations/cohorts, that lateral plate ultrastructure in threespine stickleback likely arises from a coupling of heritable and plastic effects that facilitate functional responses to the selective predation landscape.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (grant number NRC 2354 to TER) and the University of Victoria. We thank L. Page, B. Gowen, R. Marx, B. Ringwood, M. Gordon, H. Down, S. Leaver, C. Kelly, N. Harris, and S. Pokorny for their support, assistance, and technical insight during this study, and J. Buckland-Nicks for early informative SEMs of *Gasterosteus* lateral plate ultrastructure.

Land acknowledgement

TER acknowledges the Council of the Haida Nation for the continued opportunity to conduct biological studies in their traditional territory.

Article information

History dates

Received: 23 December 2021
Accepted: 27 April 2022
Accepted manuscript online: 30 May 2022
Version of record online: 27 July 2022

Copyright

Author information

Author ORCIDs

Debra L. Wertman https://orcid.org/0000-0002-8492-976X
Thomas E. Reimchen https://orcid.org/0000-0002-5578-8531

Author notes

Present address for Debra L. Wertman is Department of Forest and Conservation Sciences, Forest Sciences Centre, The University of British Columbia, 3041-2424 Main Mall, Vancouver, BC V6T 1Z4, Canada.

Competing interests

The authors declare no competing interests.

References

Ab Ghani, N.I., Herczeg, G., and Merilä, J. 2016. Effects of perceived predation risk and social environment on the development of threespined stickleback (*Gasterosteus aculeatus*) morphology. Biol. J. Linn. Soc. **118**(3): 520–535. doi:10.1111/bij.12783.

Aleyev, Y.G. 1977. Nekton. Dr. W. Junk, The Hague, the Netherlands.

Bell, M.A., and Foster, S.A. 1994. Introduction to the evolutionary biology of the threespine stickleback. *In* The evolutionary biology of the threespine stickleback. *Edited by* M.A. Bell and S.A. Foster. Oxford University Press, Oxford, UK. pp. 1–27.

Bourgeois, J.F., Blouw, D.M., Koenings, J.P., and Bell, M.A. 1994. Multivariate analysis of geographic covariance between phenotypes and environments in the threespine stickleback, *Gasterosteus aculeatus*, from the Cook Inlet area, Alaska. Can. J. Zool. **72**(8): 1497–1509. doi:10.1139/z94-198.

Breheny, P., and Burchett, W. 2017. Visualization of regression models using visreg. R J. **9**(2): 56–71. doi:10.32614/RJ-2017-046.

Bruet, B.J.F., Song, J., Boyce, M.C., and Ortiz, C. 2008. Materials design principles of ancient fish armour. Nat. Mater. **7**(9): 748–756. doi:10.1038/nmat2231. PMID:18660814.

Day, T., Pritchard, J., and Schluter, D. 1994. A comparison of two sticklebacks. Evolution, **48**(5): 1723–1734. doi:10.1111/j.1558-5646.1994.tb02208.x.

Deagle, B.E., Jones, F.C., Absher, D.M., Kingsley, D.M., and Reimchen, T.E. 2013. Phylogeography and adaptation genetics of stickleback from the Haida Gwaii archipelago revealed using genome-wide single nucleotide polymorphism genotyping. Mol. Ecol. **22**(7): 1917–1932. doi:10.1111/mec.12215. PMID:23452150.

Deagle, B.E., Jones, F.C., Chan, Y.F., Absher, D.M., Kingsley, D.M., and Reimchen, T.E. 2012. Population genomics of parallel phenotypic evolution in stickleback across stream-lake ecological transitions. Proc. R. Soc. B Biol. Sci. **279**: 1277–1286. doi:10.1098/rspb.2011.1552.

Ebenstein, D., Calderon, C., Troncoso, O.P., and Torres, F.G. 2015. Characterization of dermal plates from armored catfish *Pterygoplichthys pardalis* reveals sandwich-like nanocomposite structure. J. Mech. Behav. Biomed. Mater. **45**: 175–182. doi:10.1016/j.jmbbm.2015.02.002.

Fish, F.E., and Lauder, G.V. 2006. Passive and active flow control by swimming fishes and mammals. Annu. Rev. Fluid Mech. **38**: 193–224. doi:10.1146/annurev.fluid.38.050304.092201.

Frommen, J.G., Herder, F., Engqvist, L., Mehlis, M., Bakker, T.C.M., Schwarzer, J., and Thünken, T. 2011. Costly plastic morphological responses to predator specific odour cues in three-spined sticklebacks (*Gasterosteus aculeatus*). Evol. Ecol. **25**(3): 641–656. doi:10.1007/s10682-010-9454-6.

Giles, N. 1983. The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer Hebridean populations of the three-spined stickleback, *Gasterosteus aculeatus*. J. Zool. **199**(4): 535–544. doi:10.1111/j.1469-7998.1983.tb05104.x.

Hartig, F. 2019. DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. R package version 0.2.6. Available from http://florianhartig.github.io/DHARMa/.

Leaver, S.D., and Reimchen, T.E. 2012. Abrupt changes in defence and trophic morphology of the giant threespine stickleback (*Gasterosteus* sp.) following colonization of a vacant habitat. Biol. J. Linn. Soc. **107**(3): 494–509. doi:10.1111/j.1095-8312.2012.01969.x.

Lees, J., Märss, T., Wilson, M.V.H., Saat, T., and Špilev, H. 2012. The sculpture and morphology of postcranial dermal armor plates and associated bones in Gasterosteiforms and syngnathiforms inhabiting Estonian coastal waters. Acta Zool. **93**(4): 422–435. doi:10.1111/j.1463-6395.2011.00517.x.

Leinonen, T., Herczeg, G., Cano, J.M., and Merilä, J. 2011. Predation-imposed selection on threespine stickleback (*Gasterosteus aculeatus*) morphology: a test of the refuge use hypothesis. Evolution, **65**(10): 2916–2926. doi:10.1111/j.1558-5646.2011.01349.x.

Lenth, R. 2019. emmeans: estimated marginal means, aka least-squares means. R package version 1.4. Available from https://CRAN.R-project.org/package=emmeans.

Loewen, T.N., Carriere, B., Reist, J.D., Haldend, N.M., and Anderson, W.G. 2016. Linking physiology and biomineralization processes to ecological inferences on the life history of fishes. Comp. Biochem. Physiol. **202**: 123–140. doi:10.1016/j.cbpa.2016.06.017.

Mann, S. 2001. Biomineralization: principles and concepts in bioinorganic materials chemistry. Oxford University Press, New York.

Marques, D., Jones, F.C., Di Palma, F., Kingsley, D.M., and Reimchen, T.E. 2018. Experimental evidence for rapid genomic adaptation to a new niche in an adaptive radiation. Nat. Ecol. Evol. **2**(7): 1128–1138. doi:10.1038/s41559-018-0581-8.

Marques, D.A., Jones, F.C., Di Palma, F., Kingsley, D.M., and Reimchen, T.E. 2022. Genomic changes underlying repeated niche shifts in an adaptive radiation. Evolution, **76**(6): 1301–1319. doi:10.1111/evo.14490.

Mazzarella, A.B., Voje, K.L., Hansson, T.H., Taugbøl, A., and Fischer, B. 2015. Strong and parallel salinity-induced phenotypic plasticity in one generation of threespine stickleback. J. Evol. Biol. **28**(3): 667–677. doi:10.1111/jeb.12597.

Myhre, F., and Klepaker, T. 2009. Body armour and lateral-plate reduction in freshwater three-spined stickleback *Gasterosteus aculeatus*: adaptations to a different buoyancy regime? J. Fish Biol. **75**(8): 2062–2074. doi:10.1111/j.1095-8649.2009.02404.x.

Planidin, N.P., and Reimchen, T.E. 2019. Spatial, sexual, and rapid temporal differentiation in neuromast expression on lateral plates of Haida Gwaii threespine stickleback (*Gasterosteus aculeatus*). Can. J. Zool. **97**(11): 988–996. doi:10.1139/cjz-2019-0005.

Rasband, W.S. 2013. ImageJ 1.46r. National Institutes of Health, Bethesda, MD. Available from http://imagej.nih.gov/ij.

R Core Team. 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from https://www.R-project.org/.

Reimchen, T.E. 1983. Structural relationships between spines and lateral plates in threespine stickleback (*Gasterosteus aculeatus*). Evolution, **37**(5): 931–946. doi:10.2307/2408408.

Reimchen, T.E. 1992. Injuries on stickleback from attacks by a toothed predator (*Oncorhynchus*) and implications for the evolution of lateral plates. Evolution, **46**(4): 1224–1230. doi:10.2307/2409768.

Reimchen, T.E. 1994. Predators and morphological evolution in threespine stickleback. *In* Evolution of the threespine stickleback. *Edited by* M.A. Bell and S.A. Foster. Oxford University Press, Oxford, UK. pp 240–273.

Reimchen, T.E. 2000. Predator handling failures of lateral plate morphs in *Gasterosteus aculeatus*: functional implications for the ancestral plate condition. Behaviour, **137**(7): 1081–1096. doi:10.1163/156853900502448.

Reimchen, T.E., Bergstrom, C., and Nosil, P. 2013. Natural selection and the adaptive radiation of Haida Gwaii stickleback. Evol. Ecol. Res. **15**(3): 241–269.

Reimchen, T.E., Stinson, E.M., and Nelson, J.S. 1985. Multivariate differentiation of parapatric and allopatric populations of threespine stickleback in the Sangan River watershed, Queen Charlotte Islands. Can. J. Zool. **63**(12): 2944–2951. doi:10.1139/z85-441.

Romer, A.S. 1933. Eurypterid influence on vertebrate history. Science, **78**(2015): 114–117. doi:10.1126/science.78.2015.114.

RStudio Team. 2021. RStudio: integrated development for R. RStudio, Inc., Boston, MA. Available from http://www.rstudio.com/.

Song, J., Ortiz, C., and Boyce, M.C. 2011. Threat-protection mechanics of an armored fish. J. Mech. Behav. Biomed. Mater. **4**(5): 699–712. doi:10.1016/j.jmbbm.2010.11.011.

Song, J., Reichert, S., Kallai, I., Gazit, D., Wund, M., Boyce, M.C., and Ortiz, C. 2010. Quantitative microstructural studies of the armor of the marine threespine stickleback (*Gasterosteus aculeatus*). J. Struct. Biol. **171**(3): 318–331. doi:10.1016/j.jsb.2010.04.009.

Spoljaric, M.A., and Reimchen, T.E. 2008. Habitat-dependent reduction of sexual dimorphism in geometric body shape of Haida Gwaii threespine stickleback. Biol. J. Linn. Soc. **95**(3): 505–516. doi:10.1111/j.1095-8312.2008.01068.x.

Vermeij, G.J. 1993. Evolution and escalation: an ecological history of life. Princeton University Press, Princeton, NJ.

Wark, A.R., and Peichel, C.L. 2010. Lateral line diversity among ecologically divergent threespine stickleback populations. J. Exp. Biol. **213**(1): 108–117. doi:10.1242/jeb.031625.

Webb, J.F., and Shirey, J.E. 2003. Postembryonic development of the cranial lateral line canals and neuromasts in zebrafish. Dev. Dyn. **228**(3): 370–385. doi:10.1002/dvdy.10385.

Wickham, H. 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag, New York.

Wootton, R.J. 1976. The biology of the sticklebacks. Academic Press, London, UK.

Yang, W., Chen, I.H., Gludovatz, B., Zimmermann, E.A., Ritchie, R.O., and Meyers, M.A. 2013. Natural flexible dermal armor. Adv. Mater. **25**(1): 31–48. doi:10.1002/adma.201202713.

Yang, W., Chen, I.H., McKittrick, J., and Meyers, M.A. 2012. Flexible dermal armor in nature. JOM, **64**(4): 475–485. doi:10.1007/s11837-012-0301-9.