Why are you so defensive? Investigating the correlation between risk-taking behaviors and defensive morphology of marine Three-Spined Stickleback, *Gasterosteus aculeatus*

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Abstract:

Defensive morphologies, spines and armor, are wide-spread across multicellular life and do not exist in a vacuum, but instead co-evolve with a host of other traits to form an overall “Armor Syndrome”. The evolution of armor systems has mainly been studied in plants and mammals. Our goal for this study is to look for armor syndrome traits in fish, focusing specifically on a marine population of the three-spine stickleback (*Gasterosteus aculeatus)*. We measured various risk-taking behaviors (activity, inspection, time inspecting, orientation, and time sheltering) and ventilatory response to predator cues and correlated these with defensive morphological traits, quantified via uCT scans. Contrary to our initial expectations, we found no significant correlation between behaviors and armor measurements, but found a significant negative correlation between spine aspect ratio and number of inspections. This lack of significant correlation is similar to observations of intra-population variance in freshwater populations.

Introduction:

Physical defenses are ubiquitous across multicellular life and come in many different forms, from the spines on cacti or on a pufferfish, to the dermal armor covering on rhinoceroses. The primary factor driving the evolution of defensive structures is predation, and may be in response to generalized predation or tied to a specific “type” of predator (Crofts & Stankowich 2021), where an organism is subjected to only one mode of predation. For example, dragonfly larvae in lakes with high levels of fish predation will develop long spines that deter predation, either via the threat of puncture or by exceeding gape size. In contrast, in lakes where the main predators are mature dragonflies that grasp to capture prey rather than bite, dragonfly larvae develop shorter spines which are harder to grasp. Conversely, in environments that host a more diverse population of potential predators, such as marine environments, there is less selective pressure for highly specialized defensive structures. Beyond predation pressure, the evolution of physical defenses can be tied to any number of additional biotic and abiotic factors, such as nutrient availability or the type of habitat (Spence *et. al* 2013).

While physical defenses may be the most obvious change in response to varied predation threats, other physiological and behavioral changes occur as well, which can include risk-moderating behaviors, and changes to metabolic rates, diet, and locomotion. These form a complex and interconnected network of traits that co-evolve with robust physical defenses, called an Armor (or Defense) Syndrome (Crofts *et. al* 2021). Defense syndromes have been widely studied in plants, highlighting the interplay between defenses like spinescence and chemical defenses, and herbivory. In animals, armor syndromes have been less well studied, and the majority of work has focused on mammals (Stankowich & Campbell 2016).

The three-spined stickleback (*Gasterosteus aculaetus*) provides us with an opportunity to explore armor syndromes in fish in an eco-evolutionary context. Although ancestrally an anadromous fish, with the retreat of northern hemisphere glaciers around 12,000 years ago populations of the three-spined stickleback invaded and established numerous independent freshwater populations throughout the northern hemisphere (Bell *et. al* 2010). The different populations have independently evolved similar phenotypes, such as reduction of dermal armor plating and less pronounced dorsal and pelvic spines, in response to similar environmental cues (Reimchen 1994, 2000).

There is an extensive body of literature on the evolution of the defensive spines and lateral armored plates in these freshwater systems, showing differences in behavior between populations, and clarifying the relationship between armor and behavior. Populations of freshwater *G. aculeatus* subjected to relatively low predation risk may lose lateral plate armor coverage and pelvic girdle complexity over time when compared with populations that have higher predation pressures. Freshwater populations with lower predation risk have reduced armor coverage, but still exhibit risk-taking behaviors (Bell *et al* 2010), and will respond to predator cues, seen in higher ventilation rates when exposed to acute stressors versus the baseline ventilation rate. However, little work has explored the link between behavior and armor in the marine population.

Our goal for this study is to explore the relationship between the defensive morphology, risk-taking behaviors, and stress ventilation rates in a marine population of *G. aculeatus*. We hypothesize that individuals with greater armor coverage, denser armor, and longer, sharper spines will exhibit greater risk-taking behaviors and exhibit lower stressed ventilation rates.

Methods:

Specimens:

We collected *G. aculeatus* from the waters surrounding Friday Harbor Labs (n = 11), with an average standard length of 5.501 +/- 0.480 cm. Most individuals were collected off the docks (n=8) with the remainder (n=3) collected via beach sein. Fish were housed in flowthrough sea tables until testing.

When seining, we also collected a single *Myoxocephalus polyacanthocephalus* (standard length ~ 14 cm) to serve as a predator for our behavioral assay. The *M. polyacanthus* was maintained in a separate flowthrough sea table than the sticklebacks. All fish were cared for following IACUC protocols (protocol # 4238-03).

Stress Response

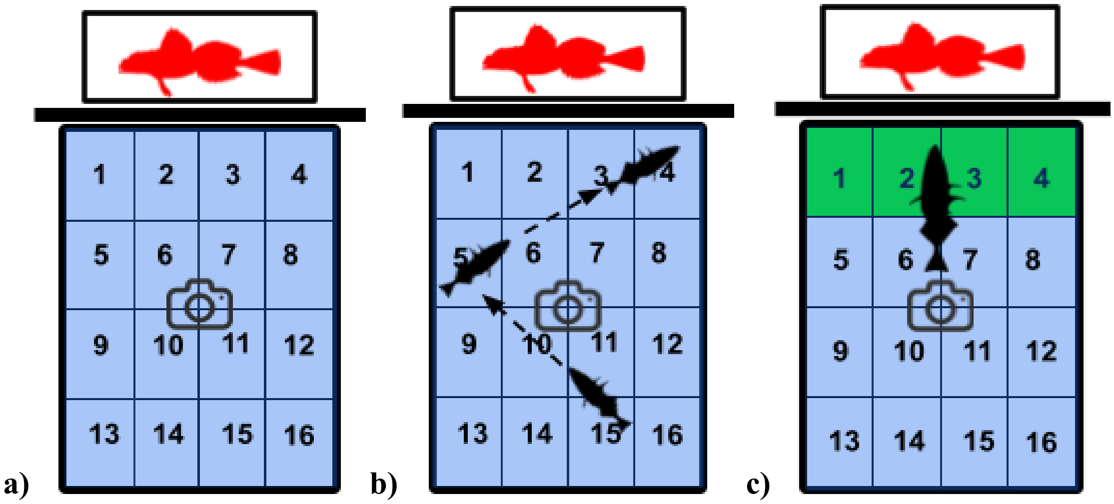
We recorded ventilation rate at two different points in our experimental process to determine if stress response varied between fish, and if we could detect any correlation with defensive morphology. We placed individuals in a small tank (1.2 L; 16 × 8.5 × 10.5 cm^3) of sea water to transfer fish from their holding tank to the experimental tank, and used this as an opportunity to assess the ventilation rate associated with a stressed condition. We filmed each fish for 1-minute in the transfer tank, counting the number of operculum beats in 15-second intervals (beats/15-seconds). To assess the baseline ventilation rate, we took a similar measure of operculum beats per 15 seconds during the final 55 minutes of the hour-long acclimation period prior to the behavioral assay.

Behavior Trials:

For our behavioral trials we subdivided the floor of the behavioral tank (20L; 51.5 ×26.5 ×30.5 cm^3; Fig. 1a) into 16 quadrants, and placed a camera (GoPro HERO10 Black) above the middle of the tank to record behavior. We wrapped three sides of the tank with black sheeting to minimize external stimuli and positioned a second tank (the predator tank; 6L; 26 ×16 ×16 cm^3) adjacent to the un-obscured fourth side. We used an opaque panel placed between the observation tank and predator tank to block the view between the two.

For each trial, following filming for stressed ventilation rate, the *G. aculeatus* was placed in the behavioral tank for a 1-hour acclimation period. About 30 minutes into the acclimation period, we introduced the *M. polyacanthocephalus* to the predator tank. Following acclimation, we introduced predator cues. To introduce a visual cue, we removed the opaque divider between the predator tank, housing the *M. polyacanthocephalus*, and the behavioral tank. At approximately the same time we added 500 mL of water from the predator tank to the behavioral tank to introduce *M. polyacanthocephalus* olfactory cues. To assess behavioral responses, we filmed the behavioral tank for 5 minutes and tracked the movement and behavior of the experimental fish.

We recorded 5 measures of behavior for each trail: 1) Activity - the number of times the *G. aculeatus* moved between sections of the behavioral tank (Fig 1b); 2) Inspection - the number of times the *G. aculeatus* swam up to the *M. polyacanthocephalus*, measured as the number of times the *G. aculaetus* entered the block of 4 quadrants closest to the predator (Fig 1c); 3) Time Inspecting - total time (seconds (s)) spent inspecting, measured as the amount of time spent in the 4 quadrants closest to the predator; 4) Time Sheltering - total time (s) spent in the last 4 quadrants of the tank furthest from the predator (Fig 1d); 5) Orienting - total time (s) spent orienting towards the *M. polyacanthocephalus* (Fig 1e).



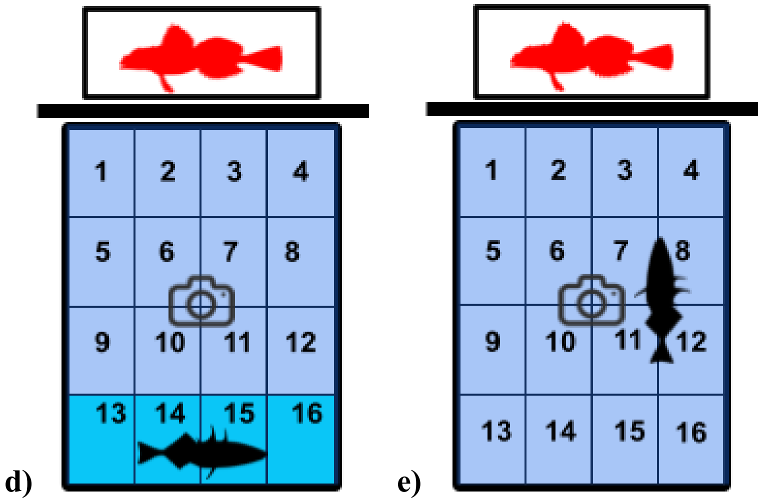


Figure 1. Trial setup with the predator tank housing the *Myoxocephalus polycanthacephalus* (red) at the top with the opaque divider between the tanks and a GoPro HERO10 Black placed above the middle of the behavioral tank (a). The behavioral tank that the *G. aculeatus* (black) is placed in is on the other side of the divider and divided into 16 quadrants. Behaviors quantified as activity (b), inspection and time inspecting (c), time sheltering (d), and orientation (e).

CT Scanning & Morphology

Following each trial, the fish was euthanized using a buffered solution of MS-222, fixed in 10% formalin for 24 hours, then transferred to a 70% ethanol solution for storage. Prior to scanning, we took lateral pictures and standard-length measurements of each specimen. We scanned fish on site at the Karel F. Liem Bio-Imaging Center (Friday Harbor Laboratories, Friday Harbor, WA), using a Bruker Skyscan 1173. We scanned in batches of 2-3 specimens at a time with a voxel size of 18.1 µm, a voltage of 65 kV, amperage of 123 µA, and an exposure of 1100 ms, using a 1mm Al filter to reduce attenuation artifacts. For each round of scanning, we also scanned two standards ("phantoms") of known densities (25% and 75% hydroxyapatite) using the same settings and resolution. All scans were reconstructed using NRecon (Bruker, 2005–2011), and in cases where phantoms were scanned separately from fish, we reconstructed phantom scans with the same settings as the corresponding batches of scanned fish to compare tissue density. We used the open-source program 3D Slicer (version r31446) with the SlicerMorph module (Kikinis et al., 2014; Rolfe et al., 2021) to isolate and measure morphology and densities.

We measured the length and maximum width of the first dorsal spine, second dorsal spines and right pelvic spine, and used these lengths to calculate aspect ratio (length/width) for each spine as a proxy for sharpness (Table 1). Included angle is the morphological measure of sharpness that most tightly correlates with puncture performance (Anderson *et. al* 2018; Crofts *et al,* 2019). While aspect ratio takes into account the entirety of the spine, rather than the morphology of only the tip, the ratio of length and width describe the included angle of the overall shape. Spines that are relatively longer for a given width, larger aspect ratios, will allow for more narrow angles at the tip. as a smaller width allows for more energy storage in a puncture event (Schofield *et. al* 2016).

To quantify armor robustness, we isolated the lateral plates in 3D Slicer, using the *Segment Editor* tool, and measured average armor plate density and percent lateral armor coverage (Table 1). To estimate lateral armor plate density, we used the 3D Slicer tool *Segment Statistics* to quantify the mean voxel brightness of the 25% and 75% phantoms. From these measures we generated standard curves describing the relationship between voxel brightness and density for each *G. aculeatus* scan. We then used the standard curves to transform the mean voxel brightness of the lateral armor plates to density in g/cm3 for each fish (Woodruff *et. al* 2022). To calculate percent lateral armor coverage, we used ImageJ (Rasband 1997) to obtain the lateral surface area of each *G. aculeatus* from specimen photos. After isolating the right lateral armor plates in 3D Slicer, we exported a screenshot with scale bar, and similarly used ImageJ to find the total surface area of the lateral armor plates. Taking the ratio of these two measurements allowed us to to calculate percent armor coverage:

$$\frac{Armor\ Surface\ Area\ (cm^2)}{Fish\ Surface\ Area\ (cm^2)} \times 100\% = Percent\ Armor\ Coverage\ (\%)$$

Data Analysis

We analyzed our data using R (version 2023.06.1+524). To test for a significant difference between stressed ventilation time intervals, we used the ANOVA function and plotted the results using the ggplot2 package. To test the relationship between the risk-taking behaviors and defensive morphological structures, we used the General Linear Models function and plotted the results using the ggplot2 package. Because all fish were of a similar size class, we did not correct for size before running our statistics.

Table 1. Spine and Armor Measurements

	First Dorsal Spine		Second Dorsal Spine		Pelvic Spine		Armor	
n	Length (mm)	Width (mm)	Length (mm)	Width (mm)	Length (mm)	Width (mm)	Density (g/cm^3)	% coverage
1	6.342	2.469	6.529	2.439	9.137	3.87	1.192786	32.38
2	5.49	1.958	5.781	1.937	9.02	3.157	1.1800326	21.45
3	6.801	2.429	7.131	2.462	10.65	3.993	1.1688908	28.39
4	4.712	1.751	5.084	1.844	7.914	2.768	1.1219392	38.2
5	6.307	2.407	6.942	2.458	9.756	3.584	1.1444372	38.85
6	5.843	1.956	6.438	1.994	8.758	3.451	1.131714	28.76
7	5.793	2.044	6.642	2.274	9.11	3.077	1.127595	39.05
8	6.558	2.194	6.752	2.213	9.867	3.192	1.1403325	31.86
9	5.722	2.134	5.901	2.032	8.635	3.413	1.13257	33.98
10	6.517	2.308	6.838	2.218	9.677	3.199	1.1147225	31.14
11	5.897	2.507	5.96	2.366	8.123	3.064	1.078775	30.78

Results:

Stress Response

The average rate of stressed ventilation was 37.05 operculum beats/15 seconds (sd = 6.52), and we found no significant difference between the 15-second periods. We found an average rate of 31.14 operculum beats/15 seconds (sd = 6.26) for the average baseline ventilation rate. We found no significant correlation between stressed ventilation and any of the defensive morphological features measured (Table 2). For baseline ventilation, we found a significant, negative correlation between the baseline and the standard length (Fig. 2).

Table 2 - Statistical results comparing ventilation and defensive morphologies

Baseline Ventilation (n = 7)				
	df	p	slope	R^2
First Dorsal Spine	5	0.631	-6.93	-0.140
Second Dorsal Spine	5	0.526	-9.524	-0.098
Right Pelvic Spine	5	0.776	4.023	-0.179
Lateral Armor Plate Density	5	0.533	-81.56	-0.102
Percent Armor Coverage	5	0.906	-0.092	-0.196
Standard Length	5	0.042	-8.767	0.515
Stressed Ventilation (n = 11)				
	df	p	slope	R^2

Baseline Ventilation (n = 7)				
First Dorsal Spine	9	0.853	-1.578	-0.107
Second Dorsal Spine	9	0.881	-1.195	-0.108
Right Pelvic Spine	9	0.183	-8.771	0.098
Lateral Armor Plate Density	9	0.336	46.41	0.003
Percent Armor Coverage	9	0.953	-0.018	-0.111
Standard Length	9	0.294	-3.352	0.024

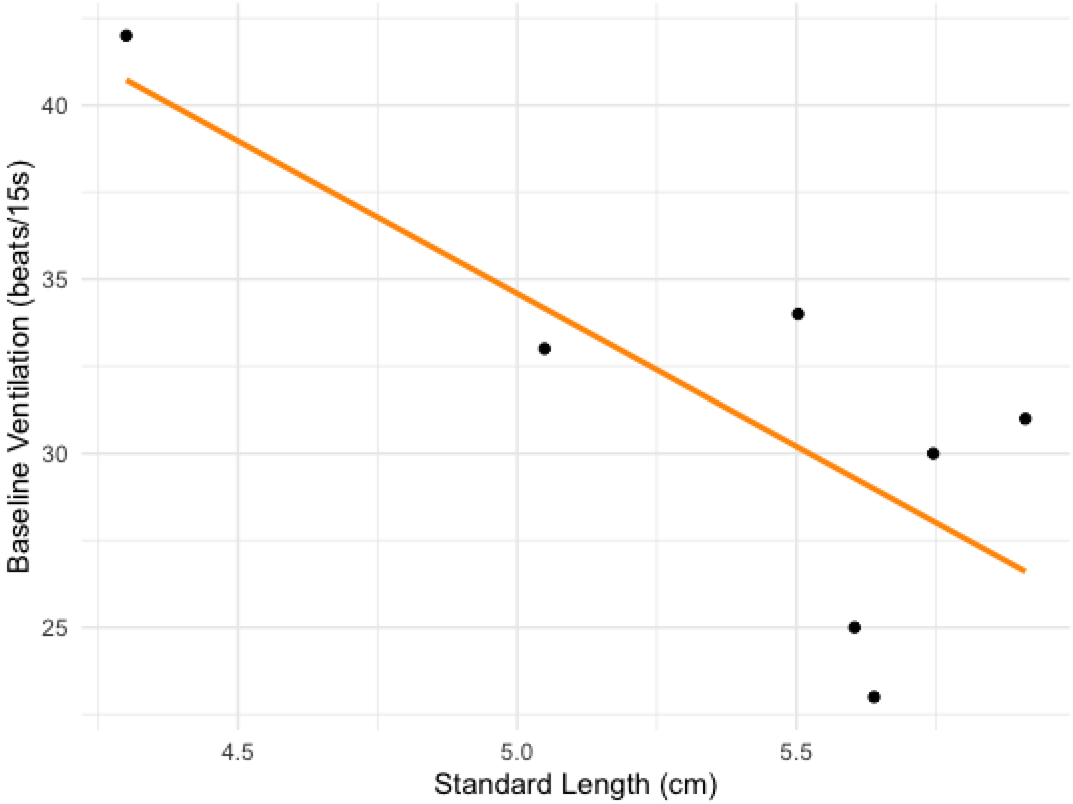


Figure 2. Significant correlation between standard length (cm) and baseline ventilation (p = 0.04198).

Behavior & Morphology

A summary of the statistical results can be found in Table 3. We found no significant correlation between activity and any of the defensive morphological structures measured. Similarly, we found no significant correlation between any of the behaviors and measures of armor robustness.

However, we found significant and negative correlations between inspection and aspect ratio for both the first and second dorsal spines, but not the pelvic spine (Fig 3). We found similar, but not significant, patterns for time inspecting and orienting.

Table 3 - Statistics comparing Risk-Taking Behaviors and Defensive Traits

Activity				
	df	p	slope	R^2
First Dorsal Spine	9	0.143	-43.56	0.136
Second Dorsal Spine	9	0.105	-44.64	0.184
Right Pelvic Spine	9	0.180	-32.77	0.1
Lateral Armor Plate Density	9	0.679	75.72	-0.089
Percent Armor Coverage	9	0.3299	-1.063	0.006
Standard Length	9	0.690	4.874	-0.090
Inspection				

	df	p	slope	R^2
First Dorsal Spine	9	0.021	-3.277	0.406
Second Dorsal Spine	9	0.014	-3.211	0.451
Right Pelvic Spine	9	0.1343	-1.883	0.146
Lateral Armor Plate Density	9	0.798	2.444	-0.103
Percent Armor Coverage	9	0.695	0.023	-0.091
Standard Length	9	0.423	-0.502	-0.030
Time Inspecting				
	df	p	slope	R^2
First Dorsal Spine	9	0.052	-103	0.287
Second Dorsal Spine	9	0.051	-97.09	0.289
Right Pelvic Spine	9	0.218	-56.64	0.0699
Lateral Armor Plate Density	9	0.9896	4.463	-0.111
Percent Armor Coverage	9	0.261	2.267	0.042
Standard Length	9	0.472	-16.2	-0.046

Time Sheltering				
	df	p	slope	R^2
First Dorsal Spine	9	0.923	-18.28	- 0.110
Second Dorsal Spine	9	0.863	-30.78	-0.107
Right Pelvic Spine	9	0.401	126.8	-0.023
Lateral Armor Plate Density	9	0.820	-249.8	-0.104
Percent Armor Coverage	9	0.553	-3.945	-0.066
Standard Length	9	0.324	70.3	0.009
Orientation				
	df	p	slope	R^2
First Dorsal Spine	9	0.094	-87.75	0.201
Second Dorsal Spine	9	0.059	-90.87	0.268
Right Pelvic Spine	9	0.262	-50.04	0.042
Lateral Armor Plate Density	9	0.536	202.4	-0.062

Percent Armor Coverage	9	0.309	1.987	0.016
Standard Length	9	0.490	-14.99	-0.051

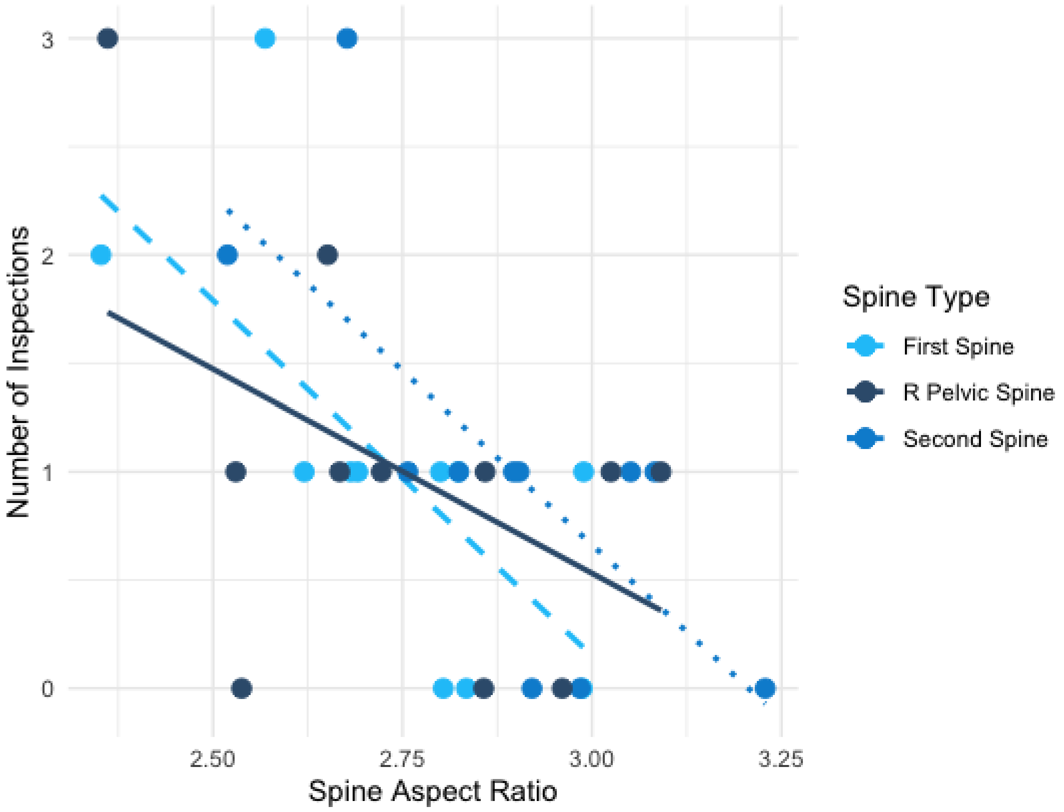


Figure 3. First and second dorsal spines significantly correlated (p = 0.0208; p = 0.01407, respectively) with the number of inspections. Pelvic spine had no significant correlation (p > 0.05).

Discussion

The only morphological defensive structures to show a significant correlation with behavior in our study were the first and second dorsal spine aspect ratio, which correlated with the number of inspections. Our initial expectations were that longer, skinner spines would serve as a sharper and a more effective puncture defense, and would be correlated with an increase in risk-taking behavior. Our observed results, however, showed a negative correlation, with fish having higher aspect ratio spines inspecting the predator space relatively fewer times than others. While time-inspecting and orienting aren't significantly correlated with the dorsal spines, the data suggest a pattern of similar negative correlation with the aspect ratios.

This observed pattern might be explained by factors we did not include in our study. The amount of energy needed to grow the longer spines may be higher than the shorter spines, increasing the overall energy needed for survival. This may cause slower metabolism in the fish with longer spines, causing them to want to spend as little energy as possible. Previous studies have shown similar results, demonstrating that individuals from populations with less developed defensive morphology, or none at all, showed more risk-taking behaviors than those with more developed defensive traits (Lacasse & Aubin-Horth 2012).

Alternatively, the correlation between dorsal spine aspect ratio and investigation could be due to predation pressure in the waters of San Juan Island. That the pelvic spine did not show significant correlation with any risk-taking behavior, while the dorsal spines did, may signal something about the most likely mode of attack this population experiences.

Contrary to our expectations, we did not observe any significant correlation, or pattern of correlation between our measures of armor robustness and any risk-taking behavior. It may be that the relatively heavy armor of the marine *G. aculeatus* population studied here limits movement, preventing the degree of behavior we expected based on similar experiments done on freshwater populations (Lacasse & Aubin-Horth 2012). For example, Northern spearnose poachers (Family: Agonidae) change their swimming pattern, from whole-body axial undulation to mainly caudal oscillation, as they grow and transition from their less-armored juvenile state to the more heavily-armored adult life stage (Mussi *et. al* 2002). In the Agonidae, this change in locomotor capability appears to be due to differences in stiffness, stemming from an increase in armor coverage. There could be a similar relation between marine and freshwater populations, where some of the freshwater populations with less armor have shown more activity and risk-taking behaviors (Lacasse & Aubin-Horth 2012; Grand 2000), meaning they are more likely to actively swim like juvenile Agaonidae, whereas the more heavily-armored marine population moves less, like the adult Agaonidae and exhibit less risk-taking behaviors.

Our results don't follow the trends previously observed in freshwater populations. Freshwater populations showed a significant decrease in stressed ventilation between each 15-second interval (Bell *et. al*. 2010), suggesting that the populations of *G. aculeatus* with lower predation pressures had reduced acute stress reactions. In contrast, our marine population of *G. aculeatus* displayed a stressed ventilation that persisted throughout the one-minute period measured. Following conclusions from freshwater studies, this would indicate that the continuous acute stress response of our marine population indicates prolonged predation risks that require longer acute stress responses.

In freshwater populations, it was observed that the *G. aculeatus* with higher stressed ventilation were more likely to exhibit risk-taking behaviors. Based on out ventialtion measurements, we might expect to see the marine sticklebacks to exhibit more risk-taking behaviors, but that was not the case in our intrapopulation study. However, there was a significant difference between freshwater populations, where the larger sticklebacks had higher stressed ventilation and exhibited more risk-taking behaviors (Bell et. al 2010). Comparatively, the marine population (standard length = 5.50 +/- 0.48 cm) is much larger than the largest freshwater population (standard length = 3.376 +/- 0.576 cm) , which would suggest that if a study was conducted comparing freshwater and marine populations, that the marine population would have higher stressed ventilation and exhibit more risk-taking behaviors (Bell et. al 2010).

This would also suggest that there may be no significant difference in a single marine population, but there could be differences between multiple marine populations.

The association of the marine *G. aculeatus* in the armor syndrome phenomena is an interesting one. Based on the previous description by Crofts *et. al* 2021, the sharper spines and more armor coverage of the marine *G. aculeatus* should allow for the population to exhibit more risk-taking behaviors, but that doesn't seem to be the case. Future studies should look at multiple different marine populations and see if there are interpopulation differences as seen between different freshwater populations.

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