What's that smell? A Whiff of Gunnel Olfactory Morphology

Ayi J. Ajavon[1], Lauren E. Simonitis[2,3]

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1- Georgia State University, Atlanta, GA
2 - Friday Harbor Labs, University of Washington, Friday Harbor, WA
3 - Florida Atlantic University, Boca Raton, FL

Contact information:
Ayi Ajavon
ayi.j.ajavon@gmail.com

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Abstract

Chemoreception is used to track predators, prey, or find conspecifics. Across the Zoarcoidei suborder, diet varies from herbivory to carnivory. Members of the Pholidae family are found along intertidal shorelines of the east Pacific ocean, and are able to breathe air during low tide. Because of these characteristics -and a knowledge gap of intertidal fish olfactory systems- we examined three species: *Apodichthys flavidus, Pholis laeta,* and *Pholis ornata*, all of which are carnivores. In our study we used contrast-enhanced microCT scans and dissections to: 1) characterize the morphology of olfactory systems; and 2) compare the olfactory morphology of two *Pholis* species to a member of the *Apodichthys* genus. We found that the two *Pholis* species have two lamellae per rosette and the average Lamellae Area scales with body length, while *Apodichthys flavidus* has up to four lamellae per rosette which do not scale with body length. Additionally, the Rosette Area of *Apodichtys flavidus* is significantly larger than both *Pholis* species. This study is the first to characterize the olfactory system of Pholids through use of microCT scanning. Further research on members of the Zoarcoidei subfamily would improve understanding on how diet may inform the level of reliance upon the olfactory system; improve our knowledge of how intertidal fishes interact with their extreme environments; and provide new information to sensory biology as a field of study.

Introduction

Even basal, unicellular organisms have evolved sensory systems to obtain information from the environment around them and use that information to make key survival decisions. The morphology of sensory systems varies throughout the different phylums; and the fishes have

evolved a multitude of sensory systems to combat intensely variable aquatic environments around the world. These senses can be sorted into two categories: 1. Short range (vision, the lateral line system and gustation) and 2. Long range (hearing and olfaction). By combining and refining these senses, fishes use them for predator avoidance, identifying potential mates, and feeding (Frade et al. 2002). The short range senses can be disrupted by many environmental factors. Vision is affected not only by habitat depth, but by turbidity. Because disturbed sediments absorb and refract light differently, a decrease in visual input causes an overall decrease in visibility, and can lower success of predator-prey interactions (Abrahams & Kattenfeld 1996, Leahy et al., 2011). Through use of the lateral line, fish can constantly detect water currents across their bodies; and can feel changes in pressure vortices and gradients as objects move around them (Bleckmann & Zelick, 2009). The proximity to a change in water movement and the energy carried through the water column is the limiting factor in the range of the lateral line system, which characterizes it as a short range sense. (Bleckmann & Zelick, 2009; Mogdens, 2019). Gustation is a relatively short range sense, and several species of fish have tens of thousands of taste buds to increase the odds of receiving an input (Hara & Zielinski, 2006). Of the long range senses, chemoreception evolved 500 million years ago as one of the earliest senses, and has the furthest range (Kajiura 2004). Unlike many of the short range senses, chemoreception is not disrupted by turbidity, nor is it overshadowed by background noise ((Abrahams & Kattenfeld, 1996; Lunt & Smee, 2015). Most fish are able to detect scents in low concentrations (some at 10^{-12} mol/liter), so only a small amount of stimuli is needed to elicit a response (Døving, 2007). In addition, reef fish larvae are known to use olfactory and lateral line inputs to detect ideal habitats for settlement (Paris et al. 2013). In aquatic environments chemoreception is reliant on odor molecules emanating from a source which could be a predator,

prey, conspecific or contaminant moving through the water column, into the nares of fish and across olfactory rosettes (Frade et al. 2002, Schlenker et al. 2019, Lari et al. 2015) . Generally, a scent molecule enters through the incurrent naris into the olfactory rosette and binds to olfactory receptor neurons (ORNs) on the lamellae. The ORN sends an action potential, starting a cascade of signals to the brain which relay information about olfactory stimuli.

The morphology of the olfactory system and lamellae may provide insight into how different species have evolved to use chemoreception (Kasumyan 2004). There is a high level of diversity in how families of fishes have evolved to direct the flow of water through the olfactory canal. Most fish have incurrent and excurrent nares, but their size, shape, and arrangement on the head varies. Some families have incurrent and excurrent nares that are widely separated on different areas of the face (e.g. Muraenidae and Bedotiidae), while others have two connected nares that act as both the incurrent and excurrent nares (e.g. Syngnathidae). Though these two olfactory canal configurations are different in appearance, their performance is very similar (Kasumyan 2004). Further diversity is revealed in the morphology of the olfactory rosette, which is dependent on the number of lamellae present and their organization. Lamellar count varies widely across families, genera and even species –with *Gambusia affinis* (of the Atheriniformes) having zero, and *Holopagus guentheri* (from the family Lutjanidae) possessing 230 (Kasumyan 2004, Pfeiffer, 1964). Lamellae are arranged parallel, bilaterally or radially –and families are usually consistent in their arrangement (Yamamoto, 1982; Kasumyan, 2004, Ferrando et al. 2019). The wide variation in anatomy and morphology of the olfactory system highlights how specialized ecological niches may be.

Overall, there is a knowledge gap in the morphology of the olfactory system in a majority of fish species and how it may be utilized for different behaviors. Though reliance on a sensory

system is difficult to quantify, morphometrics of sensory systems and brain regions can be used as a proxy for these conclusions. The olfactory system morphology of *Anguilla anguilla* (the European eel) has been described to have nearly six times as much surface area as the retina, indicating that they may be more reliant on olfaction than vision (Atta, 2013). In Carcharhinid and Sphyrnid sharks, the lamellar surface area and the distance between nares increase with Total Length, which indicates that the olfactory senses may become more complex with maturity, as these sharks are able to pinpoint the origin of scents (Kajiura et al. 2004). Aligning with other studies, an increase in olfactory bulb size is correlated to increased body length. Chondrichthyans of various trophic levels, feeding strategies, and water column habitats have different olfactory bulb arrangements –*Hemiscyllium ocellatum* (the epaulette shark) has bean shaped bulbs; *Dipturus polyommata* (the argus skate) has thick rod shaped bulbs; and *Sphyrna lewini* (the scalloped hammerhead shark) have elongated crescent shaped bulbs (Yopak 2015). The morphological differences in olfaction across taxa are wide, which could infer differences in and reliance upon olfactory capabilities.

Chemosensation is reliable in habitats where other senses are limited by turbidity, noise and high-energy waves. The rocky-intertidal is a brutal habitat that hosts all of these characteristics in addition to periods of sun and air exposure during low tide. Family Pholidae (commonly referred to as gunnels) are found across coldwater, rocky-intertidal regions of the Pacific and are very common along Northeastern Pacific coastlines, where they are found living in various seaweeds and on rocky substrates (Sweetser 2016). During low tide, gunnels find shelter by wedging their slender bodies under rocks, where they can breathe air directly and prevent desiccation and suffocation (Martin and Bridges, 1999). We are curious how the olfactory system morphology of Pholids may indicate their reliance upon it for feeding in a harsh

environment, and if these morphological traits change throughout ontogeny. To maintain uniformity, our three selected gunnel species in this study are carnivorous throughout their development and feed upon small crustaceans and other invertebrates, so we expect that their olfactory morphology would be similar (Hart, 1973; Wilkie, 1966) . The goals of our study are: 1) characterize the morphology of olfactory systems in three gunnel species (*Apdodichthys flavidus*, the penpoint gunnel; *Pholis laeta*, the crescent gunnel; and *Pholis ornata*, the saddleback gunnel) of the East Pacific; 2) investigate how olfactory organs scale with Total Length; and 3) compare the olfactory morphology of two *Pholis* species to a member of the *Apodichthys* genus.

Methods

A. Specimen Collection

Three species (*Apodichthys flavidus, Pholis laeta,* and *Pholis ornata*) were collected in tide pools along the coast of San Juan Island, Washington, USA and brought to University of Washington Friday Harbor Laboratories. Total Lengths ranged from 7.1 - 29.1cm (Table 1). Fish were euthanized in a 0.5M Tricaine mesylate (MS-222) solution then moved to a 10% formalin solution for preservation of tissue. For each fish, we collected Total Lengths before decapitation. Heads were removed slightly anterior to the pectoral fins.

Table 1: Information on morphometrics of each species collected

Scientific Name	Common Name	Number of Individuals	Min. Collected Length	Max Collected Length	Average Length
Apodichthys flavidus	Penpoint Gunnel	6	11.6 ±7.0 cm	29.1 ± 7.0 cm	16. 8 ± 7.0 cm
Pholis laeta	Crescent Gunnel	22	7.4 ± 2.23 cm	14.9 ± 2.23 cm	10.3 ± 2.23 cm
Pholis ornata	Saddleback Gunnel	18	7.1 ± 2.01 cm	13.4 ± 2.01 cm	10.4 ± 2.01 cm

B. Contrast enhanced microcomputed tomography (microCT)

After a one hour water bath, heads of each individual were placed into Phosphomolybdic acid (PMA), a contrast enhancing staining agent which increases the density of soft tissues. Each specimen was scanned using a Bruker SkyScan 1173 (Micro Photonics Inc., Allentown, PA) set to 123kV and 65A, and 1200mR exposure. Each head was scanned at 6-8µm magnification to give a high-resolution image. We used 3D Slicer (http://www.slicer.org; Federov et al., 2012) and the SlicerMorph extension (Rolfe et al., 2021) to render three dimensional compilations of each CT scan. Three axial slices which showed lamellae the best were analyzed in ImageJ (Schneider

et al., 2012) to collect rosette and lamellar area. Lamellae were numbered from proximal to distal from the naris.

C. Dissections and Scanning Electron Microscopy (SEM)

One individual of each species was dissected and imaged under a ZEISS SteREO v20 Discovery microscope (Zeiss Oberkochen, Germany) to obtain high resolution images of the olfactory system. Heads were dissected to expose olfactory rosettes for imaging. Following gross dissection, the nasal area containing the rosettes was excised and placed in an ethanol dehydration series (50%, 70% 85%, 100%) for an hour in each concentration, then placed in hexamethyldisilazane (HMDS) for further desiccation. Samples were mounted on metal SEM stubs with carbon tabs, sputter coated with gold-palladium (SPI Sputter 12121, SPI Supplies/Structure Pro, West Chester, PA), and placed into an SEM (Neoscope JCM-5000, Tokyo, Japan) for imaging.

D. Statistical Analysis

Average Lamellae Area and average lamellae was plotted against Total Length (cm^2) in a linear regression model for each of the three species used in this study. The area of the first and second lamellae were plotted by species with allometric adjustments using the “GroupStruct” R package (Chan & Grismer, 2021). ANOVA tests were used to determine significance of results with an p-value of 0.05.

Results

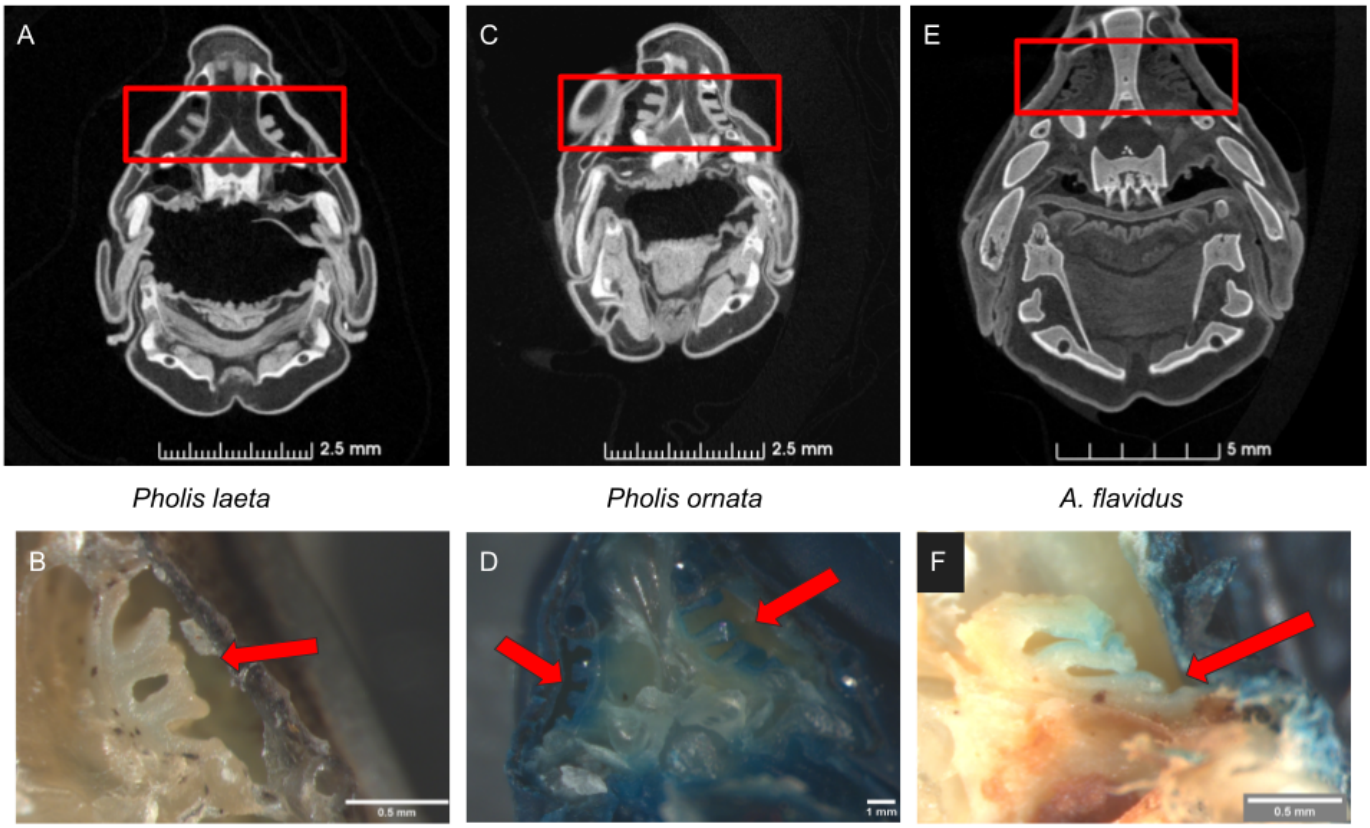

Figure 1: Visualization of gunnel olfactory rosettes.
MicroCT slices (top) and gross dissection images (bottom) of P. ornata olfactory rosettes (A,B), P. laeta (C,D), and A. flavidus (E,F). All lamellae had the same shape but varied in number. Red boxes and arrows indicate lamellae.

Pholis laeta and *Pholis ornata* have 2 lamellae per rosette while *Apodichthys flavidus* individuals had 3-4 lamellae. The two largest *A. flavidus* (TL= 18.93cm and 29.10cm) had 4 lamellae per rosette while all smaller individuals had 3. All lamellae observed had a bulbous shape with a characteristic indentation in the middle **(Figure 1)**.

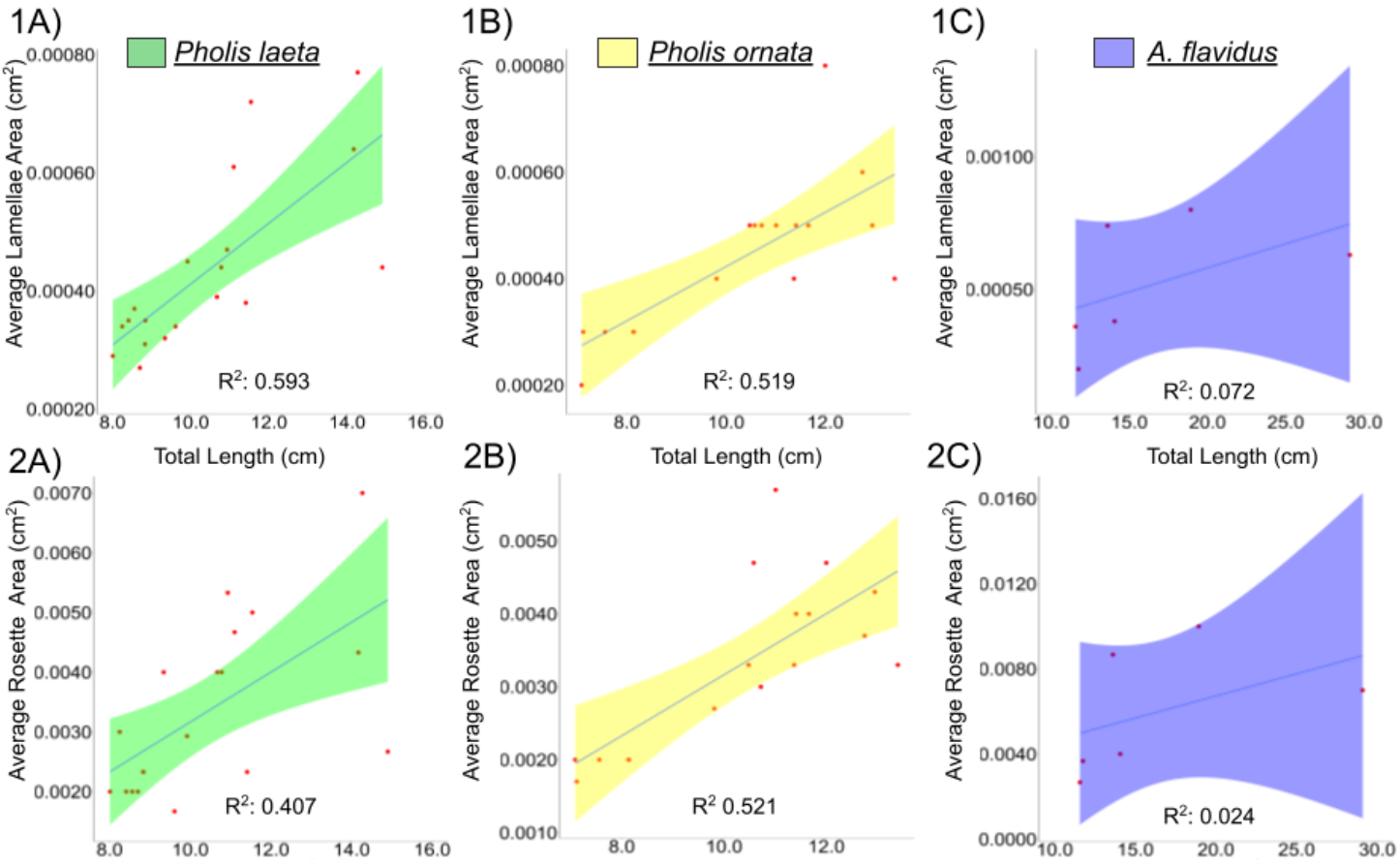

Figure 2: Olfactory morphometrics with Total Length. Linear regressions for Average Lamellae Area vs. Total Length **(1A-1C)** and Average Rosette Area vs. Total Length **(2A-2C)**for *Pholis laeta* **(A)**, *Pholis ornata* **(B),** and *Apodichtys flavidus* **(C).**

In *Pholis laeta,* Total Length accounted for 59.3% of variance for Lamellae Area and 40.7% for Rosette Area. In *Pholis ornata,* Total Length explained approximately 52% of the variance in both lamellae and Rosette Area. Total Length in *Apodichthys flavidus* was not explanatory for the variation in either traits - only 7.2% in lamellae and 2.4% in rosettes (**Figure 2)**.

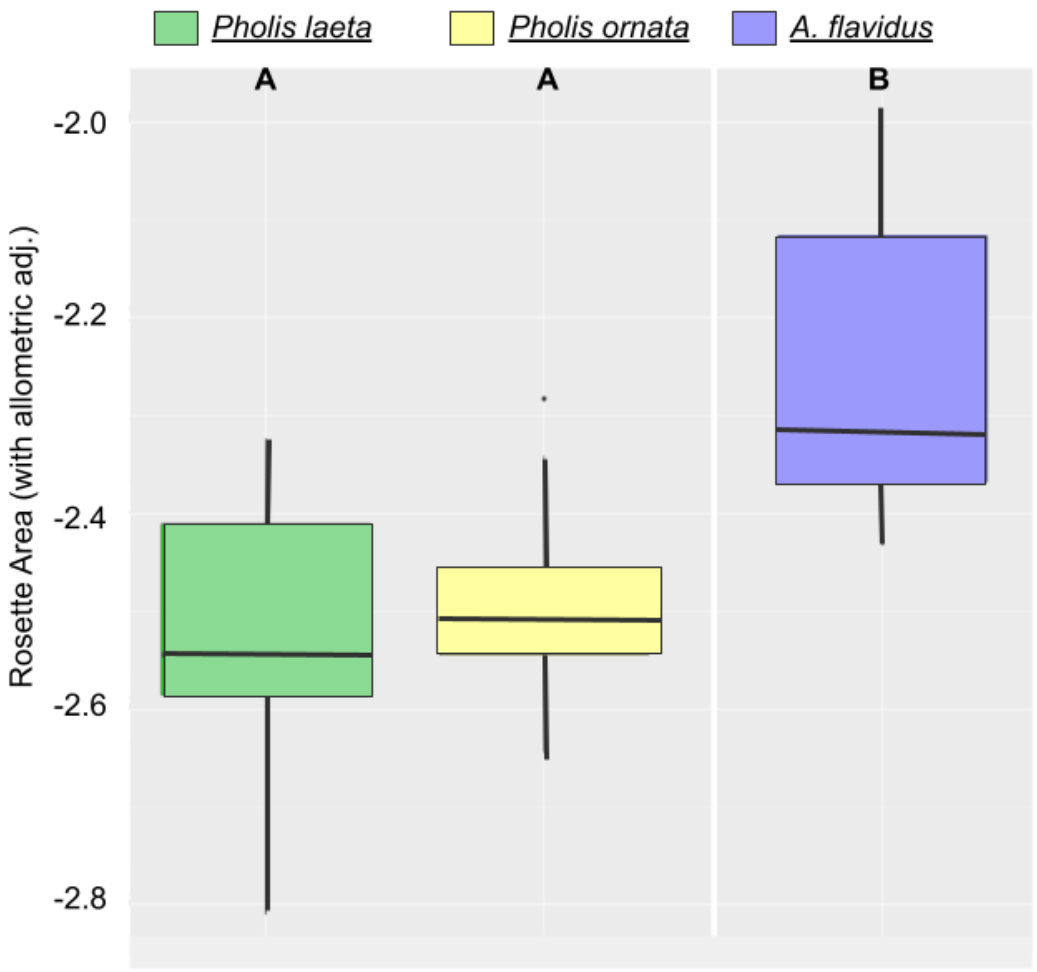

Figure 3: Allometrically adjusted Rosette Areas. Allometric scaled comparison of the area of the first and second lamellae of each species. *Pholis laeta* (green) *Pholis ornata* (yellow) *Apodichthys flavidus* (blue).

To account for variation in Rosette Area due to Total Length, Rosette Areas were allometrically adjusted. This process seeks to correct for ontogenetic variation in each species by scaling the Total Length and measured traits (in this case, rosette and Lamellae Area) in all individuals to the same size, then analyzing the variation. The Rosette Area of *A. flavidus* was significantly larger ($p<0.05$) than both *P. laeta* and *P. ornata*. There was no significant difference in rosette size between P. laeta and P.ornata ($p>0.05$; **Figure 3**)

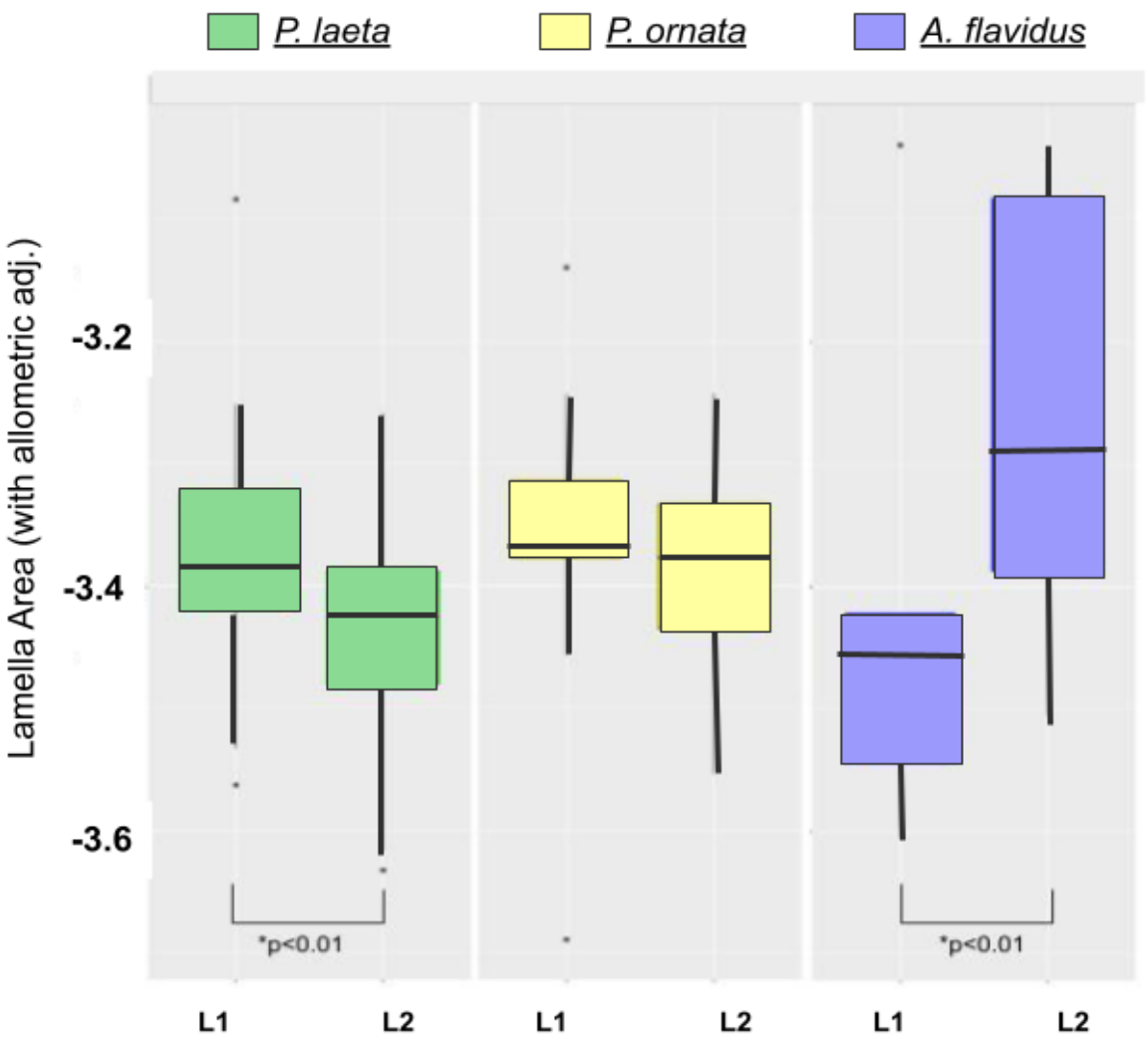

Figure 4: Allometrically adjusted Lamellae Areas. Allometrically scaled comparison of the area of the first and second lamellae of each species. *Pholis laeta* **(green)** *Pholis ornata* **(yellow)** *Apodichthys flavidus* **(blue)**. Lamellae 1 **(L1**) was the most proximal, followed by lamellae 2 **(L2**)

Allometric scaling of the area of the first and second lamellae shows significant variation in *P. laeta* and *A. flavidus* ($p<0.01$). In A. flavidus, the area of the second lamellae was larger than the first. There was no significant difference in Lamellae Area in P.ornata ($p>0.05$, **Figure 4**).

Discussion

Gunnel species differ in olfactory morphology. While all three species have the same shape of lamellae and olfactory rosette, we saw differences in lamellar arrangement. *P. laeta* and *P. ornata* had 2 lamellae per rosette, while *A. flavidus* had 3-4. In *A. flavidus*, only the two largest individuals had 4 lamellae. The largest *A. flavidus* collected was 29.1cm, though the maximum recorded length of the species is 46cm. The largest individuals the *Pholis* in our study (14.9cm for P. laeta and 13.4cm for P. ornata) were about half of the maximum recorded length of adults: 25cm and 30cm respectively (Eschmeyer et al. 1983). Because we were unable to collect individuals closer to the maximum size, it is possible that *Pholis* olfactory rosettes may also increase in lamellae number at their largest size.

We also saw interspecific differences in how olfactory morphology scaled with size. Although lamellae number increased with size in A. flavidus, there was not a strong linear relationship between Total Length and either Rosette Area or Lamellae Area. We did, however, see this pattern in both *Pholis* species which aligns with previous studies on carps (Pashchenko & Kasumyan 2017) and sharks (Kajiura 2005). Though some uncertainty remains due to our small sample size, our results show *Apodichthys flavidus* does not scale with body length. However this may be due to unobserved differences in feeding behavior or habitat preference, but further research is required. Through intraspecific comparisons of lamellae 1 and 2, we see that *Pholis laeta* displays a pattern that the first lamellae is significantly larger than the second. *Pholis ornata* displays this same trend, though it was not statistically significant. However, the opposite trend was observed for *Apodichthys flavidus* and the second lamellae is significantly larger than the first. Typically, the highest water velocities are at the opening of the nostril, so we would expect that the lamellae here would be the most robust –we see this trend in the two

Pholis species. It is peculiar that this pattern is not also observed in *Apodichthys flavidus*. Future research should investigate if water flow is different here, and if the sensory epithelium here has more surface area than in the *Pholis*. which may indicate less reliance on the olfactory system.

Understanding the morphology of olfaction may provide more insight into the ecology of gunnels and how they may use this sense for homing and feeding, which has been observed in *Cottoidea*, another family of fish common to the rocky intertidal (Craik 1978). This study is the first to our knowledge to specifically characterize the olfactory system of Pholids and of any member of the *Zoarcoidei* subfamily. By quantifying the morphometrics of other members of the *Zoarcoidei* subfamily we hope to incorporate and understand how feeding strategy and species-specific habitat preference may inform the level of reliance upon the olfactory system. Further, we would like to histologically examine olfactory systems to characterize the sensory and non-sensory epithelium. Because all three of our studied species have relatively few lamellae, we hypothesize that there is a large surface area of sensory epithelium on the rosettes, which would explain a relationship between lamellae count and sensitivity to scents. Many families in the Zoarcoidei subfamily are laterally compressed and are described as having eel-like bodies. This may mean that because the volume of the olfactory system is constrained by this body plan, lamellae counts and configurations are not as high or complex as found in other orders of fish (Kajiura et al., 2004). There are many hypotheses that could incorporate feeding behavior and strategy across the Zoarcoidei subfamily. This would widen our knowledge of phylogenetic diversity, taxonomy and potentially evolutionary history of fish in the rocky-intertidal habitat.

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