# MULTIMODAL ECOLOGY OF STICKLEBACK SENSING

By

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# A DISSERTATION

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#### ABSTRACT

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Organisms use different modes of sensing to mediate behaviors in population and community interactions. Each of these senses faces selective pressures that enable survival and reproduction in these interactions, within the environments in which they occur. Although each sensory modality may be given situational priority, multiple modalities are used in conjunction to meet an individual's informational needs in many situations. The ecological and evolutionary influences that pressure species to depend on multiple types of senses has been a subject of growing research.

The threespine stickleback (*Gasterosteus aculeatus*), a species of small fish found throughout the northern hemisphere, is a subject fit for many such investigations. Sticklebacks radiated into a multitude of freshwater habitats from ancestrally marine environments and have thus evolved to face diverse selection pressures from biotic and abiotic sources. By examining differences within and among populations from different environments, I examine how the behavioral ecology of a model species has shaped the interactions of different sensory systems.

First, I use anatomical measures of visual, olfactory, and mechanosensory systems to compare how animals invest in different modalities depending on where they evolved or how they were reared. I find evidence of population differences that show populations either invest more heavily in eyes or in olfactory tissues, at the expense of the other sense. This work thus quantifies the interactions of different sensory modalities across populations. I then study the use of visual and olfactory systems both independently and in combination as sensory modes that facilitate joining social aggregations. Though the interpretations of the full data are in progress, preliminary findings indicate that sticklebacks rely on visual over olfactory cues, but do not combine the two in a social affiliation task. However, populations differ in the magnitude of these preferences, and interactions may be of significance among fish with different habitats. This work reveals the ways in which the visual system dominates olfaction in social affiliation.

Next, I study the use of olfactory information to discriminate potential mates within and across benthic and limnetic ecotypes. Our evidence suggests that parallel evolution has resulted in the use of olfactory information as a component of mate decisions in benthic, but not limnetic, ecotypes. However, at least some limnetics show evidence of being sensitive to chemical stimuli. This work demonstrates roles of olfaction in behaviors that maintain reproductive barriers.

Finally, the use of visual and olfactory senses alone and in combination are investigated in responding to a food stimulus when in the presence of humic acids, an environmental agent potentially disruptive to both modalities. I find fish are most responsive to olfactory stimuli, and this modality is most affected by humic acids. I also find evidence that fish reduce chemical sampling for food cues in the presence of humic acids, when relying on either sensory modality alone.

In carrying out these studies, I find varying levels of support for hypotheses about the role of environment on the evolution and ecology of a model species. Further investigation of these areas will deepen understanding about how sensory systems are used in and across different species and habitats.

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#### Introduction

Sensory ecology is the study of how organism acquire and respond to information (Dusenbury 1992). Although sensory ecology has ancient roots, with consideration of differences of sensory systems among individuals, species, and sensory channels comprising elements of Greco-Roman philosophy (Martin 2017), the study of these differences as a scientific field of investigation only began to take form following Darwin and Wallace's theory of evolution by means of Natural Selection (Darwin 1859). Perceptual systems play a necessary part in the survival and reproduction of species. Tinbergen's (1952) work in animal communication placed a new emphasis on understanding signal content, the "message" of a stimulus, over the stimulus's physical properties. Combined with Lythogoe's (1979) pioneering research into the role of environmental processes that affect information detection, this would provide the grounds to develop sensory ecology as a field devoted to understanding the ecological and evolutionary functioning of sensory systems.

The same year that Dusenbery published his text defining sensory ecology, Endler (1992) put forward the suitably named Sensory Drive Hypothesis, which has since been a driver of the field (Ryan and Cummings 2013; Cummings and Endler 2018). In brief, the model stipulates that characteristics of sensory systems constrain the properties of traits that may be used as criteria for mate choice: that which cannot be detected, or is ineffectually detected, will not be selected for. These communication traits and the sensory systems that they interact with are also shaped by the surrounding environment. This is because they must convey information to the intended receiver under conditions that must be transmitted through the abiotic medium, and the information may encounter unintended receivers, such as rivals, predators, and prey. The central principle of this hypothesis is that detection of traits, through both the properties of the stimulus

and receiving sensory system, have been selected for by the physical and biological properties of the environment in which they perform. Investigating selective pressures under the framework of the sensory drive hypothesis has provided insightful answers to the functions and mechanisms of sensory systems in multiple studies for many different species and across a number of different modes of sensory perception.

However, many of the studies which have tested and found evidence for the sensory drive hypothesis have made an omission that is widely becoming acknowledged as a key gap in the field of sensory ecology. In most cases, research on how stimuli are perceived and utilized has been conducted on individual sensory channels, yet there is a growing prevalence of investigation into the role of multimodal perception. Sired in the field of animal communication (Partan and Marler 1999), investigations of multimodal perception have developed around a frame work of discerning the relationship of different sensory stimuli, whether each sensory stimulus provides the same (redundant) or different (non-redundant) information, and how the use of multiple stimuli differs from the use of senses in isolation. In this work, I use the threespine stickleback (*Gasterosteus aculeatus*) to explore how multiple sensory systems evolve across different environmental conditions

The utility of the stickleback as a study system comes from its adaptive radiation. Like many species, it originated in the world's oceans (Fang et al. 2018) and many populations still reside in marine habitats today. From what evidence is available in the fossil record, these populations have been heavily conserved; fossils of pre-historic sticklebacks are morphologically similar to modern day residents (Michael A. Bell et al. 2009). But through one way or another, the stickleback has managed to migrate to a number of estuaries and streams, rivers and lakes, and various habitats therein. And as it has migrated into these environments, it has evolved. Today,

we bear witness to a raceme of independent, repeated, and often rapid adaptations of the stickleback to similar types of aquatic habitats throughout its Holo-arctic distribution (Bell and Foster 1994). These adaptations have led to a great diversity of morphologies and behaviors in different populations, which extend into the stickleback's sensory biology.

Extensive literature exists regarding the stickleback visual system. Aspects of the stickleback's visual capabilities have been characterized down to the molecular level (Rowe et al. 2004; Rennison et al. 2016; Marques et al. 2017). The seasonal alteration of colors by males as a nuptial signal has resulted in particular attention to color sensitivity. As the male signal evolves in response to properties of the ambient environment (typically in ways that promote detectability with respect to light conditions of the local habitat), perception of these colors by females have similarly evolved, resulting in a great deal of diversity in the fish's color sensitivity across populations (Boughman 2001; Scott 2001; Rick et al. 2011). Stickleback foraging, social, and reproductive behaviors have been shown to have visual foundations, leading this to be considered the dominant modality of the species (Wootton 1976).

As a such, despite evidence for the role of olfaction in stickleback behavior (Segaar et al. 1983), research on the role of the olfactory system of sticklebacks has long lagged behind that of vision. Compared to many teleost species, the olfactory system of sticklebacks is relatively simple (Wootton 1976). Sticklebacks lack the elaborate rosettes of the epithelia tissue seen in other fish (Teichmann 1954). However, since the turn of the twenty-first century, there has been a growing interest in the role of olfaction in the species' ecology and evolution. Evidence for the use of olfaction in a variety of tasks, including recognition (Webster et al. 2007b), foraging (Webster et al. 2007a) and mate choice (Kozak et al. 2011), while widespread in the family (McLennan 2003), is disparate across populations. Interestingly, olfactory use corresponds to the visual

environment; fish from benthic (Rafferty and Boughman 2006), turbid (Quesenberry et al. 2007), tea-stained (Hiermes et al. 2015) or otherwise visually occluded habitats show a reliance on olfaction, while those from visually unobscured environments do not.

The lateral line system has received less attention than either vision or olfaction in the stickleback, although information on this system is accruing (Honkanen 1993; Wark and Peichel 2010; Jiang et al. 2015, 2017; Planidin and Reimchen 2019). Mechanosensitive neuromasts are a feature of most aquatic vertebrates (Montgomery et al. 1995). While the lateral line system is frequently divided into a pressure-sensitive canal system and velocity-sensitive superficial system, the superficial system has been lost in a number of species and may have evolved in response to habitats with slow moving water (Engelmann et al. 2000, 2002). This is the case in the threespine stickleback, and presumably happened before or shortly after the species originated, given the presence of both systems in related species (Honkanen 1993). Studies have demonstrated that the receptors of the lateral line system of threespine sticklebacks vary across habitats (Wark and Peichel 2010) and that this trait correlates strongly with a fish's ability to respond to mechanical stimulation from the environment (Jiang et al. 2015, 2017).

In this work, I expand on the existing knowledge of the stickleback's sensory ecology to explore how these different sensory systems have changed in response to evolutionary influences across populations. First, I quantify interactions between the visual, olfactory, and mechanosensory systems. I use measures of sensory morphology to determine how these systems cooperate or oppose one another. This work shows how multiple sensory systems differ in populations adapted to, or reared in, different ecological conditions.

Next, I explore the role of individual and combined sensory inputs in stickleback behavior. Using an association assay, I compare how different populations use visual, olfactory, or the

combination of sensory cues to interact with conspecifics. This work determines whether vision or olfaction is variably used in fish from different environments, and how they prioritize different sensory cues.

I then ask if the use of olfaction across populations has resulted from parallel evolution. Using populations of sympatric benthic and limnetic sticklebacks, I assess female mate preference using olfactory cues. This work demonstrates conserved patterns of olfactory use because of ecological adaptation, but also provides new evidence of olfactory perception in fish that do not use odors for detecting mates.

Finally, I investigate how visual and olfactory systems function in the face of an environmental disturbance. I use humic acid, a natural and artificially produced chemical to determine the ability of these senses to facilitate foraging and how the combination of senses may compensate for information loss when environments change.

Together, these works explore the behavioral roles of multiple modes of information acquisition, in multiple ecological contexts for a model species of fish. This provides insight on how different sensory channels are combined or prioritized, and environmental variables that may induce these relationships.

In subsequent chapters, "we" is used to describe work completed under the guidance of my advisor, Janette Boughman, and with the assistance of those who helped perform these projects.

Chapter 01 Variation in the sensory space of threespine stickleback populations In review as: Mobley, R.B. & Boughman, J.W. 2020. Variation in the sensory space of threespine stickleback populations. Integrative and Comparative Biology

#### Abstract

The peripheral sensory systems, whose morphological attributes help determine the acquisition of distinct types of information, provide a means to quantitatively compare multiple modalities of a species' sensory ecology. We used morphological metrics of the visual, olfactory, and mechanosensory lateral line sensory systems of *Gasterosteus aculeatus*, the threespine stickleback, to compare how sensory systems vary in animals that evolve in different ecological conditions, and when reared in environments that differ in the transmission of sensory stimuli. We hypothesized that the dimensions of sensory organs and correlations among sensory systems vary in populations adapted to marine and freshwater environments, and have diverged further among freshwater lake-dwelling populations. We also tested the hypothesis that the environmental conditions in which an individual develops lead to plastic differences in the morphology of sensory organs. Our results showed that between environments, fish differed in which senses are relatively elaborated or reduced. Littoral fish had larger eyes, more neuromasts, and smaller noses than pelagic or marine populations. We also found differences in the direction and magnitude of correlations among sensory systems for populations even within the same habitat type. Conditions experienced during rearing had comparatively little effect on the morphology of these senses, suggesting little plasticity under these conditions. We conclude that visual, olfactory, and lateral line systems differ among populations, in a manner that suggests genetic differentiation more than plasticity-induced variation.

#### Introduction

While used for many behavioral tasks, including foraging, predator avoidance, and communication, each sensory system responds to only certain forms of information (e.g., light, chemicals, electricity) in a manner that is highly dependent on the environment through which information is transmitted (Dusenbury 1992; Bradbury and Vehrencamp 2011; Stevens 2013). Evidence shows that sensory systems have high metabolic and cognitive costs (Niven and Laughlin 2008; Moran et al. 2015), and consequently, we may see tradeoffs where the elaboration of one sensory modality is accompanied by the regression of another modality that cannot function well in that environment. This appears to explain why many subterranean and troglodyte species that operate in low light environments often show enhanced chemical and tactile systems in combination with extreme regression or even complete loss of the visual system (Catania 2005; Yoshizawa et al. 2012). Tradeoffs predict negative correlations among senses. Despite the potential for tradeoffs, all but the most extreme specialists rely on multiple types of sensory information (Partan and Marler 1999; Ward and Mehner 2010; Higham and Hebets 2013; Partan 2017; Swafford and Oakley 2018). Different senses may provide redundant or distinct types of information that are combined by a receiver to obtain more reliable messages, or may evoke different responses than information obtained from a single modality. Moreover, properties of the environment can simultaneously affect more than one information channel. For example, organic acids in the water can affect both the chemical and photic properties of the environment (Secondi et al. 2015; Mobley et al. 2020), by changing pH and the absorbance of short wavelength light. As such, in many lineages we may expect to see positive associations of distinct sensory systems responding to environmental conditions. Thus, the senses that are used may be influenced by multiple, intertwined, biotic and abiotic factors of a species' ecology (Endler 1992).

We studied how environmental conditions affect multiple sensory organs in the threespine stickleback (Gasterosteus aculeatus). This fish species originated in the ocean (Fang et al. 2018), and still possesses many marine resident populations, but also has come to inhabit various estuaries, streams, rivers, and lakes of the Northern hemisphere (Bell and Foster 1994). Populations from similar ecologies show similarity in their sensory systems (Boughman 2001; Wark and Peichel 2010; Mobley et al. 2016), implicating selection as a key driver of sensory properties. In addition, plasticity of developmental patterns in response to environment may also shape sensory biology, as occurs in other traits (Hofmann et al. 2010; Chapman et al. 2010; Wund et al. 2012). The well-studied stickleback visual system has shown shifts in sensitivity to light intensity across much of its visual spectrum as freshwater populations evolved from marine environments (Rennison et al. 2016). In several populations, visual sensitivity has been demonstrated to align with properties of the physical and biological visual environment (Boughman 2001; Rick et al. 2011). Interestingly, the use of olfaction seems to be negatively correlated with vision at the behavioral level: sticklebacks from benthic (Rafferty and Boughman 2006; Mobley et al. 2016), turbid (Engstrom-Ost and Candolin 2007) or otherwise visually restrictive environments (Hiermes et al. 2015) show a greater use of olfaction than those from more visually facilitative habitats. The number of neuromasts, the receptor organs of the mechanosensory lateral line system, also differs across habitats; stream sticklebacks possess more of these sensory organs than neighboring marine populations, and benthic sticklebacks possess more neuromasts than sympatric limnetic fish (Wark and Peichel 2010). While the three sensory systems investigated here do not comprise the totality of the species' sensory modalities (McLennan 2007), the adage that form follows function holds for the sensory systems; visual, olfactory and mechanosensory organ structures are influenced by the environments in which they

evolve and develop (Montgomery et al. 1995; Kasumyan 2004; Hofmann et al. 2010; Chapman et al. 2010; Nummela et al. 2013; Cronin et al. 2014; Weiss 2019).

We used fish from marine and freshwater pelagic and littoral populations to explore how sensory systems have changed in the sticklebacks' radiation from marine to freshwater environments. We anticipated that substantial differences in the visual environment would drive the evolution of multiple sensory modalities, as non-visual senses appear to be dominated by the visual sense in this species (Wootton 1976). To represent the ancestral marine sticklebacks, we collected anadromous fish from Bonsall Creek. Marine stickleback are found in well-lit open oceanic environments most of the year and migrate into estuaries and rivers to breed (Rennison et al. 2016). Bonsall Creek is a shallow, sparsely vegetated creek that empties into an estuary, both of which the stickleback inhabit during the breeding season. We obtained fish from the creek and the estuary. Stickleback occupation of freshwater habitats has resulted in extensive morphological character evolution, and these adaptations often reflect particular types of habitat (Vines and Schluter 2006; Gow et al. 2008; Matthews et al. 2010b). We used published data on body shape and nuptial coloration of fish, and ecological information on the nature and abundance of vegetation, prey type, and visual transmission properties of waterbodies themselves (Ormond et al. 2011, personal observations) to describe freshwater populations as being from littoral or pelagic habitats. Beaver and Brannen are small, heavily vegetated lakes, resulting in a tannin-stained light environment (Boughman 2001), and sticklebacks are large with deep bodies and low nuptial coloration in males, a trait that evolves in visually restricted environments (Scott 2001). As such, these populations were categorized as littoral. Priest Lake resides in an alkaline basin with a deep shelf. While Priest Lake contains sympatric populations of limnetic and benthic sticklebacks, in this work we used only benthic fish. We categorized

these fish as littoral, in accordance with their bottom feeding lifestyle, and the similarity of their morphological features (i.e., deep bodies, few gill rakers, reduced nuptial coloration in males, and similarities of size and shape) to other littoral populations (Vines and Schluter 2006). While Weston is also a small vegetated lake, it has low to moderate levels of turbidity and clear water color (Ormond et al. 2011), and sticklebacks in Weston Lake are small, slim and show vivid nuptial coloration during the breeding season. Therefore, Weston fish were categorized as pelagic, along with fish from Sproat Lake. Sproat is a large, deep lake of high water transparency, and the size and shape of Sproat fish is similar to that of limnetic populations (Vines and Schluter 2006).

In addition to comparing fish from different natural lakes, we asked whether sensory systems show plasticity by manipulating visual and chemical conditions experienced during fish maturation to emulate properties of the environment thought to affect the evolution of sensory traits. Evaluating plasticity allows us to infer whether any differences we find in natural populations result from genetic and/or environmental effects.

We measured morphological attributes of the visual, olfactory, and mechanosensory systems of threespine sticklebacks from different natural environments and reared in different artificially altered environments. From these attributes we constructed sensory spaces, a conceptual presentation used to describe and compare how senses differ and interact among environments, populations, or species. The morphology of a sensory system has functional impacts, and there are also biomechanical and physiological constraints to the use and development of multiple senses (Aiello and Wheeler 1995; Herrel et al. 2009). By using morphological dimensions of sensory organs as a proxy of investment in a sensory system, a sensory space depicts an estimate of the relative roles of senses, and the correlations and trade-offs between them, for particular

phylogenies and ecologies (Nummela et al. 2013). The sensory space concept is analogous to color space which highlights spectral tuning and can be used for comparison (Guerrieri et al. 2005; Cronin et al. 2014). To explore how the dimensions of sensory systems relate to their environment, we tested if morphological metrics of sensory systems have changed in the transition from marine to different freshwater environments. We predicted that the high amount of visual information found in pelagic habitats, and rearing conditions with abundant light and chemical environments that restricted transmission of chemical stimuli, would reduce the need for other types of sensory information and result in larger eyes, fewer neuromasts in the lateral line and smaller noses, as well as a stronger, negative correlation between visual and nonvisual senses than seen in other habitats. Littoral populations, and fish that develop in light-restricted habitats, were expected to incorporate both chemical and mechanosensory information to compensate for limited visual information, and so were expected to show smaller eyes, more neuromasts and larger noses, as well as a positive correlation among nonvisual modalities, and a negative correlation between vision and these other senses. Our work quantified the relationships among multiple sensory systems and how these varied due to the sensory environment in nature, and in the lab.

## Methods

Animal Collection and Housing

We obtained sticklebacks from six locations in British Columbia, Canada. Animals were collected from Bonsall Creek, and from Beaver, Brannen, Priest and Weston Lakes in 2015, and again in 2017 (except from Priest) with the addition of fish collected from Sproat Lake.

All fish were gathered using minnow traps and then transported and housed in tanks at Michigan State University based on population. Animals were fed *ad libitum* daily a mixture of frozen

bloodworms and brineshrimp until their use in data collection. Measurements of the eye, nose, or number of neuromasts were taken from 209 fish (Table S1.1A).

# Morphological Measurements *Neuromast Counts*

To quantify the mechanosensory system, we counted the number of neuromasts of the lateral line system, which detect water movement around a fish's body (Jiang et al. 2017). The procedure for quantification of the lateral line system was modified from Wark and Peichel (2010). Fish were stained in a 0.25 g/L solution of DASPEI (2-[4-(Dimethylamino)styryl]-1-ethylpyridinium iodide; Sigma Aldrich 3785-01-1) for 15 minutes, then sedated in aerated 0.25 g/L MS-222, buffered to neutral with sodium bicarbonate for one minute, and then placed in buffered 0.125 g/L MS-222 for two minutes. The animal was placed in a petri dish containing 0.125 g/L MS-222 and viewed under a Leica fluorescence microscope fitted with an ET-GFP filter (Figure 1.1A). Neuromasts of each area of the lateral line system on one side of the body were counted once by a single observer, and photographed with a Leica DM700 camera. Fish were euthanized by over exposure to MS-222 following neuromast counts for subsequent measurements.

#### Eye and Body Length/Shape Measurements

We quantified the visual system by measuring the anterior-posterior diameter of one eye, as this dimension of the eye influences visual acuity (Green et al. 1980). We also measured body length and shape, as these traits may influence the dimensions of all the sensory systems. The body was blotted dry, the abdomen squeezed gently to remove digesta (and eggs in gravid females), and photographed. Morphological landmarks on pictures of fish were placed using tpsDig (Rohlf 2006), from which eye diameter and body length were measured as Euclidian distance between specified marks (Figure 1.1B). To assess differences in overall body morphology between habitat types on sensory biology, we performed a Principal Components Analysis (PCA) of recorded

body landmarks. For wild fish, an analysis of variance (ANOVA) on the first principle component axis, which explained 21.5% of the variation in body shape, found a significant effect of habitat type ( $F_{2,206} = 30.9$ , p < 0.001). Using habitat type as the grouping variable, we then performed a between-group PCA (package "Morpho", Schlager 2017) on the landmarks and used individuals' scores on the first between-group axis, which explained 16.1% of the variation as the measure of shape.

#### Olfactory Epithelial Measurements

We measured the area of the olfactory epithelium to quantify the olfactory system, as the size of the epithelium constrains the number of receptor cells of the olfactory system (Kasumyan 2004) and thus may affect olfactory sensitivity. Histological examination of the olfactory system followed the protocols described in Humason (1962). In brief, first the anterior of the cranium between the upper lip to just before the eyes was removed with a pair of dissection scissors and placed in a labeled tissue cartridge. The tissue was then fixed in Bouin's solution overnight (VWR 4539464). The specimen was then decalcified in 25% Formic Acid A for 48 hours, dehydrated in a series of ethanol baths, cleared in toluene, and embedded in paraffin under vacuum. The specimen block was then cut in 20 micrometer thick sections on a microtome, mounted on gelatin-subbed slides and heated on a slide warmer, and left to dry at room temperature overnight. Slides were stained in Harris Hematoxylin (VWR 95057-844) and Eosin (Thermo-Fisher 90049) after Humason (1962). To measure the size of the olfactory epithelium, every fifth section of tissue, starting at the first detection of olfactory epithelial tissue, was photographed under a microscope. Photographed sections were measured in ImageJ via transverse marking (Figure 1.1C) and multiplied by the section thickness. The size of unmeasured sections was interpolated by averaging the measured sections and multiplying by the

thickness of the unmeasured sections. These interpolated values were combined with measured values to provide the overall measurement of the olfactory area (Rosen and Harry 1990).

#### **Experimental Rearing Conditions**

To evaluate the extent of plasticity, Priest benthic fish collected in 2015 were artificially crossed to produce clutches that were split among environmental and dietary treatments during rearing, and so any differences found in sensory morphology would be the result of environment, rather than genetics. Briefly, artifical crosses were done by gently stroking a female's abdomen to release eggs. Males were euthanized and testes removed and macerated, and then sperm from a single male was used to fertilize eggs of a single female (Martinez et al. 2016). Clutches were then split in half, with half the offspring raised in an environment designed to facilitate vision and inhibit olfaction, and the other half raised in an environment designed to inhibit vision and facilitate olfaction. In what we refer to as the "Full" environment, fish were reared under standard laboratory lighting via fluorescent bulbs in water buffered to pH 6.5 using Arowana Buffer (Seachem Laboratories, Madison, GA). Subjects reared in the "Full" environment had access to greater amounts of light over a broader spectrum with more acidic pH which inhibits olfactory sensitivity of aquatic vertebrates (Muller-Schwarze 2006a). In the "Red" environment, the light was shifted via gel filters on light sources, which only allowed the transmission of long wavelength light (GamColor G250 Medium Red XT, Rosco Laboratories Inc. Stamford, CT), and buffered to a slightly alkaline pH of 8.5 with Tanganyika Buffer (Seachem Laboratories, Madison, GA). Conditions in the "Red" environment were those thought to promote olfaction, but restrict visual sensing (Moore 1994; Scott 2001). In each treatment, after the larval period during which fish were fed on hatched brineshrimp, subjects were kept on a diet either of exclusively frozen bloodworms (macroinvertebrates typical of a littoral diet) or frozen brineshrimp (a planktonic prey type typical of a pelagic diet) until their use in data collection.

Measurements of the eye, nose, and the number of neuromasts were taken from 88 fish (Table S1.1B) using the methods described above. For these fish, an ANOVA on the first principle component axis of landmarks (which explained 24.7% of the variation in body shape) revealed a significant effect of the light environment ( $F_{1,84} = 13.6$ , p < 0.001), but not diet or the light-diet interaction. Using rearing environment as the grouping variable we performed a between-group PCA on the landmarks and used individuals' scores on the between-group axis, (which explained 18.7% of the variation), as the measure of shape.

#### **Statistical Analysis**

All analyses were carried out in R (R Core Team 2017). To compare differences among individual sensory systems (i.e., the number of neuromasts in each of the 12 divisions of the lateral line, the total number of neuromasts, eye diameter, and the olfactory area), we used linear modeling to assess the role of population, body length and shape for wild fish, and the effects of light and diet treatments, body length and shape for experimental fish. Including body length in the linear model accounts for size effects on sensory measures. Each sensory measure was first fitted to a linear model including all interactions. We evaluated the use of specifying a variance structure by population with Akaike Information Criteria (AIC), and likelihood ratio tests. In wild fish, models of olfactory area, total number of neuromasts, and counts of neuromasts in most of the individual regions of the lateral line were significantly improved when stratifying the variance by population, so we elected to specify a variance structure for all variables to facilitate comparisons of different response variables. We elected not to specify a variance structure for lab-reared fish because the only significant improvements in model fit came from stratifying the variance by the light-diet interaction for the SO and ET divisions of the lateral line. We used AIC and likelihood ratio tests to perform backward model selection, and ultimately used analysis of

covariance (ANCOVA) on the main effects only models with type II sums of squares to analyze model terms. Contrast tests with a false discovery rate correction (FDR), based on comparisons between habitat types of wild fish, and between rearing environments for lab fish, were used to test for differences between populations using the package "predictmeans" (Luo et al. 2018).

We determined the correlations among sensory systems with Pearson's correlation tests for fish that received all measures of eye diameter, olfactory area, and the total number of neuromasts, (limited by fish for which olfactory samples were obtained: 124 for wild fish, 58 for experimentally reared fish). Differences in correlations for different habitats or rearing environments were compared using Fisher's r-to-Z transformations of independent groups using the package "cocor" (Diedenhofen and Musch 2015).

While absolute sensory organ size has functional implications, because each of these variables is correlated with body size, in comparing the relationships of different modalities to each other, we corrected for body size. Body size is always considered, but in two different ways. First, with body length as a covariate for mean estimates of individual modalities (above), Second, we regressed each sensory measure against body length, and used the residuals of these regressions to compare the differences and relationships of different size-adjusted sensory organs (referred to as "residual" measurements). Results of multivariate analyses and correlations between unadjusted sensory organ measures are reported in the supplement. Multivariate analysis of covariance (MANCOVA) was used for measures of the residual eye diameter, olfactory area, and the total number of neuromasts to evaluate the effects of population (or rearing treatments for the lab fish), body shape, and their interaction on combined senses.

## Results Wild fish

Sensory Space

Populations of sticklebacks from different habitats varied in measurements of individual sensory organs and in the residual correlations among different sensory systems. Eyes of littoral fish were significantly larger than those of either pelagic or marine populations, though marine and pelagic populations did not differ from one another (Figure 1.2, Figure S1.1A). Marine fish possessed larger noses than freshwater populations, and the noses of pelagic fish were larger than those of littoral fish (Figure 1.2, Figure S1.1B). Littoral fish possessed more total neuromasts than either marine or pelagic populations, and marine fish had more neuromasts than pelagic fish (Figure 1.2, Figure S1.1C).

In the different individual regions of the lateral line system, marine fish had significantly fewer neuromasts than littoral fish in seven lines, but more neuromasts than pelagic fish in 5 lines. Littoral fish had significantly more neuromasts than pelagic fish in eight lines. Neither CF nor AP differed among populations (Figure 1.2, Figure S1.1D).

We used size-corrected residuals for all correlations. We found a significant negative correlation between olfactory area and the diameter of the eye in all populations (Pearson's r = -0.35, p < 0.01; Table 1.1A). This effect was most pronounced in pelagic fish, for which this correlation was significantly more negative than the correlation in littoral populations (Fisher's Z = 2.13, p < 0.05; Table 1.1B).

Except for one positive correlation, in Beaver Lake, the correlation between residual olfactory area and the total number of neuromasts was not significant in any individual population or habitat type (Table 1.1A). The correlation of residual olfactory area and neuromast number for littoral and pelagic populations was significantly greater than for the marine population (Table

1.1B). The residual nose-line correlation did not differ significantly between freshwater habitat types.

There was a significant positive correlation between residual eye diameter and the total number of neuromasts in marine fish and a significant negative correlation between these parameters in littoral populations (Table 1.1A). The correlation was significantly greater in marine than freshwater fish collectively, and specifically for littoral populations, but not pelagic populations (Table 1.1B). The residual lateral line-eye correlation did not differ significantly between littoral and pelagic populations.

Overall, Bonsall (the marine population) and Sproat (a pelagic population) were very similar to each other in all three modalities and separated in sensory space from littoral populations and the pelagic Weston population on the visual and olfactory plane. Littoral and Weston fish were characterized by relatively large eyes and olfactory tissue, while Sproat and Bonsall fish grouped at the lower ends of these trait values. Within the grouping of littoral and Weston fish, populations exhibited further variation in the non-visual axes. The pelagic Weston population had similar numbers of neuromasts as Bonsall and Sproat, and the littoral Brannen population had similar nose size. On average, littoral fish had the largest residual eyes, smallest noses, and most neuromasts; pelagic fish had intermediate size eyes and noses and the fewest neuromasts; and marine fish had the smallest eyes, largest noses, and an intermediate number of neuromasts (Figure 1.3).

## Population, size and shape effects on sensory morphology

The size of the eye was significantly correlated with body length and shape, and differed significantly across populations (Table S1.2). The size of the olfactory epithelium was significantly influenced by body length, but not shape, and differed significantly across

populations (Table S1.2). The total number of lateral line neuromasts was not significantly affected by either body length or shape, but did differ among populations (Table S1.2). In the individual divisions of the lateral line shape had no significant effect and body length was significant only for the count of neuromasts in the IO region. However, population differences were significant for most regions (Table S1.2). We found a significant effect of population on residual measures for the three sensory systems taken together (MANCOVA, approx.  $F_{15,336} = 12.2$ , p < 0.001) but no effect of shape, nor an interaction between shape and population.

### Experimental fish and plasticity

We found limited evidence of plasticity due to rearing environment. The residual size of the nose and number of neuromasts for subjects overall showed a significant positive correlation. No other correlations were significant (Table 1.2). We found no significant differences in the size of the eyes, olfactory epithelium, or number of neuromasts (in individual divisions or overall) across rearing environments or dietary treatments (Figure S1.2). Though not significant, on average, fish reared in the "Red" environment had a trend of larger residual eyes, noses and more neuromasts than those raised in the "Full" light environment (Figure 1.4).

Body length was a significant predictor of eye size, nose size, similar to wild fish, and also the number of neuromasts for lines of the trunk (Mp and Ma), and the posterior-lateral head (ST, OT, and PO) as well as the overall number of neuromasts (Table S1.3) which contrasts with wild fish. Shape significantly affected eye size and the number of neuromasts in the CF and OT lines, but did not significantly affect total neuromast count or nose size (Table S1.3).

### Discussion

The sensory space concept is intended to describe and reveal cooperation and tradeoffs among senses that might be shaped by ecological and evolutionary processes (Nummela et al. 2013). Illustrating where in multidimensional sensory space a population or species resides reveals

which sensory modalities predominate, and comparing across populations subject to different selective regimes can highlight sensory evolution. As vision may be strongly selected for due to its many utilities in visually facilitative environments, in visually restrictive environments other senses may be expected to be prioritized at the cost of vision. We had predicted that freshwater fish would have diverged from marine sticklebacks in sensory morphology, and that pelagic populations would prioritize visual over non-visual senses, with the opposite occurring in littoral populations (Niven and Laughlin 2008; Yoshizawa et al. 2012; Cummings and Endler 2018). We did find differences among populations in their sensory spaces. However, the sensory space occupied by individual populations did not follow the marine, littoral, and pelagic habitat classifications we gave populations based on prior data for body morphology, nuptial coloration, and attributes of lake ecology (Reimchen 1989; McKinnon 1995; Scott 2001; Vines and Schluter 2006). Rather, we found sensory groupings that reflected other physical attributes of the waterbodies that these populations originated from, such as size and depth of waterbodies. This suggests that selection acts on sensory traits differently than on body shape or coloration, and that the sensory environment may vary along different axes than other aspects of the ecological niche. The sensory environment of Sproat Lake may be similar to open water marine environments due to its great size and depth, unvegetated water column, and high water transparency. The position of Weston fish close to littoral populations in sensory space may reflect the abiotic similarities of Weston to Brannen and Beaver as they are all small shallow lakes (but see supplemental discussion). Larger noses have been reported in more pelagically adapted species relative to those from benthic habitats in elasmobranchs and are hypothesized to be an adaption to capitalize on the scarcity of cues in this type of environment (Schluessel et al. 2008).

While our results concerning marine sticklebacks bear judicious interpretation since they are represented by a single population, the comparisons to freshwater habitats made here pose intriguing perspectives and questions regarding the evolution of these senses in sticklebacks. Because sticklebacks have invaded multiple types of freshwater environments at multiple independent times in history (Bell and Foster 1994), the direction and extent of body morphological evolution manifests in different directions across populations. We see this in sensory morphology of all three sensory modalities as well, though each undergoes different paths. Eye size is larger in freshwater than marine habitats, for both littoral and pelagic fish. The pattern of increasing eye size in freshwater environments may come with the altered transmission of light and reduced irradiance of freshwater, relative to marine, environments (Rennison et al. 2016). This reduced irradiance of freshwater light environments may select for relatively larger eyes of freshwater fish to increase light sensitivity, that we find here, and may also be matched by changes in the relative expression of opsins to complement the relative intensity of the corresponding light spectrum of the ambient environment (Rennison et al. 2016). (Boughman 2001; Scott 2001).

In the evolutionary transition from marine to lacustrine ecosystems, fish encountered more variation in the light and chemical environments. The presence of a large nose in marine sticklebacks seems to support the idea that the use of olfaction is ancestral, and the limited use of olfactory information seen in some populations is a derived condition (Rafferty and Boughman 2006; Kozak and Boughman 2009; Hiermes et al. 2015; Mobley et al. 2016). However, it is surprising that we found littoral populations, which in these prior studies are the users of olfaction, are those that have relatively smaller noses. Although larger noses are often associated with olfactory specialists and benthic ecotypes, more elaborate olfactory organs have been found

in some pelagic and coastal elasmobranchs (Schluessel et al. 2008; Timm and Fish 2012). Dimensions beyond epithelial size alone may inform how the olfactory sense is employed by sticklebacks, which in some populations have been shown to alter their use of olfaction depending on the visual environment, and further exploration of this modality in different behavioral contexts is needed (Engstrom-Ost and Candolin 2007; Heuschele et al. 2009).

Considering that marine fish only displayed an intermediate number of neuromasts it appears that this is the only sensory system to diverge in different directions in littoral and pelagic freshwater habitats. Furthermore, variability in the number of neuromasts is not uniformly distributed across the body; the number of neuromasts in our populations was conserved between freshwater habitats around the eye and in the medial-dorsal head area. Even though surprisingly few studies have related the distribution of neuromasts to their functionality, modeling of physical activity of water with neuromast physiology indicates that the location of neuromasts concentrated on the body influences how fish respond to hydrodynamic stimuli (Ristroph et al. 2015). Differences in the trunk and posterior-lateral head may reflect the functional regionalization of the lateral line, and we find that marine fish differ from littoral and pelagic populations in the number of neuromasts around different parts of the head. Neuromasts of the anterior are thought to be particularly important for responding to flow variation from the surrounding environment and be used in foraging (New 2002; Ristroph et al. 2015; Mogdans 2019), perhaps explaining why littoral fish have more neuromasts near the jaw and eye than marine or pelagic fish. The posterior neuromasts may have greater roles in social behaviors like shoaling or mate choice (Partridge and Pitcher 1980; Satou, Takeuchi, Nishii, et al. 1994; Satou, Takeuchi, Takei, et al. 1994). Yet, littoral fish have been shown to have reduced shoaling compared to marine populations, despite their numerous main trunk line neuromasts (Greenwood

et al. 2013). It should also be noted that marine fish possess multiple, highly developed bony plates along the body, unlike our lake populations, which have reduced plate number and size. Lateral plates influence the arrangement and number of neuromasts of the main posterior trunk region (Mills et al. 2014; Jiang et al. 2017; Planidin and Reimchen 2019).

The morphology of an individual sensory systems may carry ecological relevance, but the relationships of multiple sensory systems with each other may further elucidate how sensory systems evolve. The existence of a negative correlation between two variables does not equate to a tradeoff, though it is often characteristic of such (Roff and Fairbairn 2007). Vision and olfaction may be expected to tradeoff because of the cost of maintaining different sensory system, or to improve performance in one modality, at the expense of the other (Niven and Laughlin 2008; Yoshizawa et al. 2012; Moran et al. 2015). We had initially expected a negative relationship between visual and olfactory sensory organs, as suggested by other studies of bimodal sensory relations (Atta 2013; Keesey et al. 2019), and as anticipated, this was pattern was found, but only in pelagic populations, and in the opposite manner than expected. The enlargement of noses and accompanying reduction of eye size found only in pelagic populations may reflect specialization enabling fish to rely on fewer senses in open water habitats (as suggested above), and implies that this is accomplished by diminishing senses that fish use less. Such sensory degradation may be a result of physiological costs to sustaining sensory organs (Niven and Laughlin 2008; Moran et al. 2015), or may occur due to reallocation of space in the telencephalon to particular senses (Sylvester et al. 2013). However, diminishing a modality may come without explicit cost, if the environments of the diminished sense are sufficient for an organism to fulfill an ecological function while using fewer resources (e.g., eyes can be small and detect light well in bright habitats, but must be large to function in dim settings). Contrary to the expectation that olfaction and mechanoreception would be positively correlated, which would suggest the two modalities have increased roles when vision is reduced, our finding that these senses rarely relate to one another suggests a functional independence of these senses, and perhaps freedom in morphological constraints (the interior nose does not directly compete with external neuromasts for space on the body (Yoshizawa et al. 2012)). But, as seen in the littoral Beaver population, these modalities may still act complementarily in some conditions, even if those are not recognizable by broad categorizations of marine, pelagic, and littoral habitats, unlike non-sensory traits such as body size and shape.

We also explored the potential for conditions experienced during rearing to affect individual senses and shape the sensory space. Rearing environments with an abundance of light and restriction of chemical stimuli were expected to result in fish with relatively larger eyes and reduced non-visual senses. But fish reared under different conditions did not significantly differ from one another in metrics of different senses, which suggests differences seen among wild populations are primarily the result of genetic differences. Although the power for this experiment was low, the correlations of the sensory systems overall are like those seen in wild fish from their parent population, from Priest Lake. While fish reared under experimental conditions did not match their parent population in terms of absolute sensory dimensions, wild caught Priest benthics also showed a strong, albeit nonsignificant, positive correlation of nose size and neuromast number, but no other sensory correlations. However, the sensory space of fish reared under the experimental conditions showed trends like those seen in the differences between wild populations. Eye size, and to a lesser extent nose size, showed the greatest disparity between rearing environments in sensory space, and the eye size-olfactory area plane was the major plane of division for wild populations. The lateral line differences better

demonstrated the dietary differences within the red environment than the other senses. In other species, it has been shown that lateral line development is constrained by phylogeny, rather than environmental conditions, suggesting that dimensions other than the number of neuromasts, such as their density, the number of neuromasts within an area of the body, may be the source of functional, adaptive variation seen across species (Higgs and Fuiman 1998). The physical properties of the treatments may have resulted in the prominence of vision and olfaction in defining the habitat differences, as was seen in the differences between wild littoral and pelagic fish. While the mechanosensory environments were consistent across treatments, differences in the lateral line may reflect indirect effects, such as the reshaping of the jaw apparatus in response to different diets (Wund et al. 2008), affecting the placement and number of neuromasts on the surface of the body. Other non-sensory traits, such as body shape and spine position have been shown to be more susceptible to environmentally induced plasticity during ontogeny (Garduño-Paz et al. 2010).

The information available to an organism is a product of the physical properties of a stimulus and the role of the stimulus in a species' ecology (Dusenbury 1992). This information is obtained through senses that receive information in multiple physical forms, but provide the means for distinct or redundant types of information to interact (Rowe 1999). In quantifying the peripheral morphology of visual, olfactory and mechanosensory systems of the threespine stickleback, we show that vision and olfaction separated populations and appear to reflect differences in the physical environment, and that non-visual senses further distinguish populations originating from small waterbodies. Alteration of the visual and chemical environments during rearing had no significant effect on the morphology of sensory systems. Multimodal systems provide numerous challenges in study, but combining the role of different senses, environmental conditions, and

informational needs are necessary endeavors for understanding the form and function of the behavioral responses coordinated by sensory systems.

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Data Availability Statement: Data from this study will be deposited according to standard field practices in Dryad prior to publication of the manuscript.
APPENDICES

### APPENDIX A



Chapter 1 Figures and Tables

### Figure 1.1 Stickleback sensory morphology measurements.

A) DASPEI stained stickleback, showing the distribution of lateral line neuromasts. Abbreviations for line names: supraorbital (SO), otic (OT), anterior pit (AP), supratemporal (ST), main trunk line anterior (Ma), main trunk line posterior (Mp), caudal fin (CF), ethmoid (ET), mandibular (MD), oral (OR), infraorbital (IO), and preopercular (PO). Image was created by superimposing pictures taken along the lateral anterior-posterior axis at different magnifications to show the positioning of neuromasts and is not to scale. B) Diagrammatic image of stickleback showing placement of landmarks used for shape quantification. (1) anterior tip of upper lip; (2) anterior extent of eye; (3) dorsal extent of eye; (4) posterior extent of eye; (5) midpoint along dorsal midline between landmarks 3 and 6, roughly lateral to supraoccipital notch; (6) end of head, roughly above 16, found by following groove from landmark 16 up to dorsal midline; (7) anterior insertion of first dorsal spine; (8) anterior insertion of second dorsal spine; (9) anterior insertion of the dorsal fin; (10) dorsal point at the narrowest part of the caudal peduncle; (11) caudal border of hypural plate at the lateral midline; (12) ventral point at the narrowest part of the caudal peduncle; (13) anterior insertion of anal fin membrane; (14) anterior junction of pelvic spine on ventral midline; (15) Anterior extent of the ectocorocoid; (16) posteriodorsal extent of operculum; (17) posterioventral extent of preopercular; (18) Dorsal extent of the preopercular; (19) Inside corner of preopercular; (20) Anterior extent of the preopercular; (21) posterior tip of upper lip; (22) Posterior extent of ectocoracoid. Measures of eye diameter are equal to the length between marks 2 and 4; measures of body length are equal to the distance between marks 1 and 11. C) A frontal section of H&E stained stickleback olfactory epithelium (outlined in black); anterior left, lateral bottom. Scale bar = 1 mm.



Figure 1. 2 Comparisons of sensory morphology between habitat types.

Inset table: t-test statistics from habitat contrasts (on models of TRAIT ~ Population + Body Length + Shape) of eye diameter (EYE), olfactory area (NOSE), the total number of neuromasts (LINE), and each individual division of the lateral line fish (two letter abbreviations; see Figure 1.1A) in wild populations. Significant (FDR adjusted, p < 0.05) values are indicated in bold. Positive values indicate a greater value of the trait for the habitat first named in the contrast. In figure, coloration indicates a significant difference in the contrast for the indicated trait for comparisons of: A) Marine vs all Freshwater populations; B) Marine vs Littoral; C) Marine vs Pelagic; D) Littoral vs Pelagic; E) All of the preceding comparisons, colored as in A-D: black indicates all comparisons were significant. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article. Schematic sticklebacks modified from Wark and Peichel 2010.



Figure 1. 3 Residual sensory space of wild populations.

Residual three-dimensional sensory space showing the mean positions of 6 populations of sticklebacks. The vertical z axis represents the residual number of neuromasts, the horizontal y axis represents the residual nose size (left scale), and the horizontal x axis represents the residual eye size (right scale). The means of each freshwater ecotypes and rearing environments are indicated by hexagrams.



Figure 1. 4 Residual sensory space of sticklebacks reared in experimental conditions.

Residual three-dimensional sensory space showing the mean positions of sticklebacks exposed to different environmental and dietary rearing treatments. The vertical z axis represents the residual number of neuromasts, the horizontal y axis represents the residual nose size (left scale), and the horizontal x axis represents the residual eye size (right scale). The means of each freshwater ecotypes and rearing environments are indicated by hexagrams.

### Table 1.1 Correlations and differences of residual sensory measures for wild populations.

Habitat/Population	Residual Eye-Nose Correlation	Residual Nose-Line Correlation	Residual Line-Eye Correlation
All Wild Fish	-0.35***	-0.13	-0.02
Marine (Bonsall)	-0.11	-0.34	0.51***
All Freshwater	-0.20	0.03	-0.16
Littoral Populations	0.04	0.14	-0.34**
Beaver	0.40*	0.45*	-0.19
Brannen	-0.19	-0.19	-0.17
PriestB	0.15	0.56	0.05
Pelagic Populations	-0.43*	0.23	0.04
Sproat	-0.70	0.13	-0.28
Weston	-0.24	-0.04	0.29

Table 1.1A Residual Sensory Correlations of Wild populations

Table 1.1B: Differences in correlation of residual sensory correlations among habitat types of wild populations.

Habitat Comparison	Residual Eye-Nose	Residual Nose-Line	Residual Line-Eye
$(7, \mathbf{score})$	Correlation	Correlation	Correlation
(Z-SCOLE)	Difference	Difference	Difference
Marine vs Freshwater	0.42	-1.72	3.25**
Marine vs Littoral	-0.62	-2.14*	3.93***
Marine vs Pelagic	1.27	-2.11*	-1.85
Littoral vs Pelagic	2.13*	-0.37	-1.72

A) Correlation coefficients by habitat and population for each combination of residual eye diameter (Eye), olfactory area (Nose) and total neuromasts (Line) for populations of wild fish. Significant correlations indicated in bold (Pearson's correlation test, r = 0; \* = p< 0.05, \*\*p <0.01, \*\*\*p <0.001). B) Fisher's Z statistic for comparisons of the correlations between residual eye diameter (Eye), olfactory area (Nose) and total lateral line neuromasts (Line) between habitats of wild fish (\* = p < 0.05, \*\* = p < 0.01; \*\*\* = p < 0.001).

# Table 1. 2 Correlations and differences of residual sensory measures for experimentally reared fish.

Table 1.2A: Residual Sensory Correlations of populations reared under experimental conditions

Rearing Conditions	Residual Eye-Nose Correlation	Residual Nose-Line Correlation	Residual Line-Eye Correlation
Full Environment	-0.22	0.56	0.54
Shrimp Diet	-0.40	0.62	0.24
Worm Diet	N/A	N/A	N/A
Red Environment	0.06	0.26	-0.15
Shrimp Diet	0.12	0.23	-0.23
Worm Diet	0.01	0.36	-0.09

Table 1.2B: Differences in correlation of residual sensory correlations of fish reared under different experimental conditions.

Habitat Comparison (Z-	Residual Eye-Nose	Residual Nose-Line	Residual Line-Eye
score)	Correlation	Correlation	Correlation
	Difference	Difference	Difference
Red vs Full	0.69	-1.85	-0.90
Red: Shrimp vs Worms	-0.34	0.46	0.50

A) Correlation coefficients by experimental rearing conditions for each combination of residual eye diameter (Eye), olfactory area (Nose) and total neuromasts (Line) for populations of wild fish. No significant (p < 0.05) correlations were found. Correlations of senses for fish reared on the worm diet in the Full environment were not calculated as we did not obtain a sufficient number of samples for all measures of eye diameter, olfactory area, and total number of neuromasts. B) Fisher's Z statistic for comparisons of the correlations between residual eye

diameter (Eye), olfactory area (Nose) and total lateral line neuromasts (Line) between experimental rearing conditions. No significant (p < 0.05) differences in correlations were found. Comparisons of diet treatments for fish in the Full environment were not calculated as we did not obtain a sufficient number of samples for all measures of eye diameter, olfactory area, and total number of neuromasts.

# APPENDIX B

Chapter 1 Supplemental Text

Supplemental Results

Correlations of sensory measurements

# Wild fish

Without accounting for body size and considering only absolute size of organs, the correlations between different sensory organs were not significant, except for a positive correlation between eye diameter and olfactory area in the Beaver population, and differences between habitat types were not significant (Table S1.4A-B).

The MANCOVA on sensory organ dimensions was statistically significant for population (approx.  $F_{15,336} = 15.30$ , p < 0.001), but not shape or the interaction term. On average for absolute size: littoral fish had the largest eyes, smallest noses, and most neuromasts; pelagic fish had the smallest eyes (though about the same size as marine), intermediate noses and the fewest neuromasts; and marine fish had intermediate sized eyes, the largest noses, and an intermediate number of neuromasts (Figure S1.3).

# Experimentally reared fish

Across all experimentally reared fish when using absolute size and not correcting for body size, the relationships of sensory organs were all positively correlated (Table S1.4C). The correlations of olfactory area with eye diameter, and neuromast number were not significant for fish reared in the full environment. Additionally, the correlation of olfactory area with neuromast number was not significant for fish in the red environment raised on shrimp (Table S1.4D). No correlations of eye diameter and neuromasts number were significant within either light or diet group.

The MANCOVA on sensory organ dimensions was statistically significant for the two-way interaction of light and diet (approx.  $F_{3,48} = 3.01$ , p < 0.05), but no other effects were significant. On average, for absolute size, fish reared in the red environment had larger eyes, noses and more neuromasts than those raised in the full environment. (Figure S1.4).

# Supplemental Discussion

# Unsized Sensory Space

Weston fish fall much closer to littoral populations in the residual sensory space, which may reflect the abiotic similarities of Weston with Brannen and Beaver. Yet in overall morphology Weston is more similar to Sproat than any other population (Figure 5), and it is notable that in the sensory space that does not account for body length, Weston and Sproat are much more closely aligned on the visual axis, but remain separated by olfactory morphology (Figure S1.4).

### APPENDIX C





Figure S1. 1 Means of sensory dimensions for wild fish.

Means of A) eye diameter, B) olfactory area and C) total number of neuromasts for populations of wild fish: Error bars denote +\- 2 standard errors of the mean. D) Mean number of neuromasts in each individual division of the lateral line system for each population of fish: Error bars denote +1 standard error of the mean. See also Figure 1.2 for habitat differences in traits, and Table S1.2 for size, shape and population effects.



Figure S1. 2 Means of sensory dimensions for experimentally reared fish.

Means of A) eye diameter, B) olfactory area and C) total number of neuromasts for fish reared under different environmental and dietary treatments: Error bars denote +\- 2 standard errors of the mean. D) Mean number of neuromasts in each individual division of the lateral line system for fish reared under different environmental and dietary treatments: Error bars denote +1 standard error of the mean. See also Table S1.3 for size, shape and experimental treatment effects.



Figure S1. 3 Sensory space of wild fish.

Diagram showing the mean positions of 6 populations of sticklebacks in a three-dimensional sensory space of absolute sensory organ measures (residual values are reported in the main text, Figure 1.3). The vertical z axis represents the number of neuromasts, the horizontal y axis represents the olfactory area (left scale), and the horizontal x axis represents the eye diameter (right scale). The means of freshwater habitats are indicated by hexagrams.



Figure S1. 4 Sensory space of experimentally reared fish.

Diagram showing the mean positions of environmental and dietary rearing treatments in a threedimensional sensory space for absolute organ measures (residual values are reported in the main text, Figure 1.4). The vertical z axis represents the number of neuromasts, the horizontal y axis represents the olfactory area (left scale), and the horizontal x axis represents the eye diameter (right scale). The means of each environmental treatment are indicated by hexagrams.



Figure S1. 5 Between group distances of body shape landmarks.

Dendrogram of the Euclidean distances for the average between group principal components of body morphological landmarks from populations of wild fish.

# Table S1. 1 Study sample sizes.

Table S1.1A: Sample Sizes for measurement	s of different sensory organs for wild
populations	

Population	Eye Diameter sample size	Olfactory Area sample size	Lateral line neuromasts sample size
Beaver	35	30	35
Bonsall	35	29	34
Brannen	35	26	35
PriestB	20	10	19
Sproat	31	6	19
Weston	53	23	35

Table S1.1B: Sample sizes for fish raised in under experimental conditions

Full Environment/ Shrimp Diet	16	8	16
Full Environment/ Worm Diet	5	2	5
Red Environment/ Shrimp Diet	33	25	33
Red Environment/ Worm Diet	34	23	33

Sample sizes of each analyses of individual sensory organs for A) wild populations and B) experimental rearing treatments. Correlations and multivariate analyses used only the samples that had measures of all three sensory systems, which was the same data as those used in univariate assessment of olfactory area.

Table S1. 2 Model effect statistics on sensory organ measures from populations of wild fish.

		EYE	NOSE	LINE	CF	Мр	Ma	ST	OT	PO	MD	OR	IO	ET	SO	AP	
	df	201	116	169	169	169	169	169	169	169	169	169	169	169	169	169	
Pop	5	73.8 5*	85.05 *	278.3 1*	31.1 *	239.8 9*	100.1 1*	123.6 8*	80.4 3*	116. 9 *	143.5 4*	75.7 *	83.2 4*	56.8 1*	149.3 6*	37.7 9*	
Size	1	230. 48*	5.57* *	0.01	3.1	0.3	0	0.02	1	0.35	0.01	0.2	4.95 **	1.49	1.85	0.29	
Shape	5	23.7 5*	0.05	0.61	2.7	0.37	0.26	0	1.11	0.34	0.07	1.77	0.61	2.09	1.14	0.62	
Chi-square test statistics for model terms from type II sums of squares ANOVA on main effects models (TRAIT ~ Population + Size +																	
Shape) of: eye diameter (EYE), and olfactory area (NOSE), the total neuromasts count (LINE), and each division of the lateral line																	
system for wild fish (Two letter abbreviations). Significant terms are in bold (* = $p < 0.05$ , ** $p < 0.01$ , *** $p < 0.001$ ).																	

		EYE	NOSE	LINE	CF	Мр	Ma	ST	OT	PO	MD	OR	ΙΟ	ET	SO	AP
	df	83	53	82	82	82	82	82	82	82	82	82	82	82	82	82
Environ ment	1	0.16	0.09	0.03	0.23	0.01	0.11	0.3	0.4	1.14	2.48	1.86	0.44	1.18	1.09	1.68
Diet	1	1.76	0.5	0.4	0	0.64	0	0.07	0.08	0.18	0.44	3.71 <sup>.</sup>	0.53	0.04	1.4	0.44
Size	1	117.5 ***	36.95 ***	8.76**	0.37	5.21*	14.1***	5.95***	6.93*	7.11**	0.69	1.06	2.73	0.36	2.48	0.51
Shape	1	16.18 ***	1.21	1.63	4.00*	1.18	2.5	0.68	4.49*	0	0.06	0.88	3.16 <sup>.</sup>	0.38	0.41	0.25
Chi-square test statistics for model terms from type II sums of squares ANOVA (models of TRAIT ~ Environment + Diet + Size +																

# Table S1. 3 Model effect statistics for fish reared under different experimental conditions.

Shape) for: each division of the lateral line system, the total neuromasts count (LINE), eye diameter (EYE), and olfactory area (NOSE) for experimental fish. Significant terms are in bold (\* = p < 0.05, \*\*p < 0.01, \*\*\* p < 0.001).

Table S1.4A Sen	sory Correlati	ons of Wild p	opulations	Table S1.4C: Sensory Correlations of populations				
Habitat/Population	Eye-Nose Correlation	Nose-Line Correlation	Line-Eye Correlation	Rearing Conditions	Eye-Nose Correlation	Nose-Line Correlation	Line-Eye Correlation	
All Wild Fish	-0.11	-0.05	0.17	All Rearing Conditions	0.54***	0.46***	0.27*	
Marine (Bonsall)	0.21	-0.11	0.48	Full Environment	0.41	0.56	0.46	
All Freshwater	-0.07	0.07	0.13	Shrimp Diet	0.17	0.67	0.39	
Littoral Populations	0.07	0.17	-0.02	Worm Diet	N/A	N/A	N/A	
Beaver	0.46*	0.34	-0.24	Red Environment	0.58***	0.43**	0.23	
Brannen	-0.01	-0.29	0.08	Shrimp Diet	0.56**	0.37	0.14	
PriestB	-0.22	0.31	-0.4	Worm Diet	0.47*	0.59**	0.37	
Pelagic Populations	-0.13	0.3	0.07					
Sproat	0.01	0.18	-0.39					
Weston Table S1 4B: Dif	0.05 ferences in co	-0.03	0.35	Table S1 4D. Di	fferences in co	orrelation of r	esidual	

# Table S1. 4 Non-residual sensory correlations of wild and experimentally reared fish.

Table S1.4B: Differences in correlation of residual sensory correlations among habitat types of wild populations.

Table S1.4D: Differences in correlation of residual sensory correlations of fish reared under different experimental conditions.

Table S1.4 (cont'd)

Habitat Comparison (Z-score)	Eye-Nose Correlation Difference	Nose-Line Correlation Difference	Line-Eye Correlation Difference	Rearing Condition Comparison (Z-score)	Eye-Nose Correlation Difference	Nose-Line Correlation Difference	Line-Eye Correlation Difference
Marine vs Freshwater	1.27	-0.83	1.77	Red vs Full	0.54	-0.41	-0.6
Marine vs Littoral	0.6	-1.2	2.35	Red: Shrimp vs Worms	-0.4	0.93	0.84
Marine vs Pelagic	1.23	-1.52	1.66				
Littoral vs Pelagic	0.86	-0.61	-0.38				

A) Correlation coefficients by habitat and populations, for each combination of non-residual measures of eye diameter (Eye), olfactory area (Nose) and total neuromasts (Line). (Residual values are reported in the main text, Table 1.1A). Significant correlations indicated in bold (Pearson's correlation test, r = 0; \* = p< 0.05, \*\*p <0.01, \*\*\*p <0.001). B) Fisher's Z statistic for comparisons of differences in correlations between non-residual measures eye diameter (Eye), olfactory area (Nose) and total lateral line neuromasts (Line) between habitats of wild fish (Differences of residual correlations are reported in main text Table 1.1B; \* = p < 0.05, \*\* = p < 0.01; \*\*\* = p < 0.001). C) Correlation coefficients by rearing conditions of experimental fish, for each combination of non-residual measures of eye diameter (Eye), olfactory area (Nose) and total values are reported in the main text (Table 1.2A)). Significant correlations indicated in bold (Pearson's correlation test, r = 0; \* = p< 0.001).

Correlations of senses for fish reared on the worm diet in the Full environment were not calculated as we did not obtain a sufficient number of samples for all measures of eye diameter, olfactory area, and total number of neuromasts. D) Fisher's Z statistic for comparisons of differences in correlations between non-residual measures eye diameter (Eye), olfactory area (Nose) and total lateral line neuromasts (Line) between rearing conditions of experimental fish. (Differences of residual correlations are reported in main text Table 1.2B; \* = p < 0.05, \*\* = p < 0.01; \*\*\* = p < 0.001). Comparisons of diet treatments for fish in the Full environment were not calculated as we did not obtain a sufficient number of samples for all measures of eye diameter, olfactory area, and total number of neuromasts.

Chapter 02: Stickleback use of visual, olfactory, and bimodal cues in social associations Abstract

Sensory systems are prioritized differently by populations that evolve in diverse environments. Many species live in ephemeral, changing groups of conspecifics and individuals join groups based on information obtained by one or several sensory modalities. Here we compare how visual, olfactory, and bimodal cues are used to form associations in populations of the threespine stickleback. Doing so evaluates which mode of sensory information is more relevant to certain populations, and which components of a bimodal stimulus are used in these decisions. Complete evaluation of this work is in progress. Here, we outline the key observations, questions, hypotheses, and predictions that motivate this work. We also provide the methodology and current results of statistical analysis, which indicate visual dominance over olfaction across populations, but demonstrate differences in populations for the magnitude of this preference. We outline these results in relation to these hypotheses, with conclusions about population differences conditional on types of stimuli as a subject of future research.

### Introduction

Environmental factors drive the evolution of sensory systems (Endler 1992). Ecological and organismal variation may promote the use of particular modes of sensing such as greater auditory sensitivity of nocturnal, compared to diurnal species (Nummela et al. 2013; Goyret and Yuan 2015). As animals may rely on multiple senses, environmental variation is expected to influence the evolution of individual as well as combinations of sensory systems (Partan and Marler 1999; Hebets et al. 2014).

The selective pressures on sensory systems may come from several sources. Transmission properties of the environments in which sensory stimuli travel, needs to detect predators and prey, and interactions with conspecifics influence how sensory systems fit a species' ecology

(Cummings and Endler 2018). Particular selection may act upon participation in social groups. Fish aggregate with members of their own population, and the sensory cues to form these aggregations will depend on the environmental properties and variation in those properties (Partridge and Pitcher 1980; Webster et al. 2007b). The ability to form social groups within an environment may depend on particular combinations of senses. Combinations of senses may counter or complement one another, or come at another's expense (Ganson 2018). Information obtained by different senses may prompt the same (redundant) or different (non-redundant) responses. Combining sensory stimuli may result in more reliable messages, or ones more easily detected by an organism (Partan and Marler 1999; Rowe 1999).

The threespine stickleback (*Gasterosteus aculeatus*), has radiated to habitats that vary in transmission properties for different sensory modalities. Vision is known to highly influence social behaviors in the stickleback, but the exact nature may vary across habitat types (Nomakuchi et al. 2009; Modarressie et al. 2015). While often considered a lesser modality in stickleback ecology, olfaction also has demonstrated roles in stickleback behaviors, and shows environmental variation (Ward 2004; Webster et al. 2007b). Here we look at population variation in the use of vision, olfaction, and the combination of the two in stickleback social affiliations.

We ask if populations vary in preference for visual over bimodal, and bimodal over olfactory cues. We hypothesize that in social association choices, stickleback adaptations lead to variation in use of visual and olfactory cues; associations with other fish are based on visual cues, rather than olfactory cues, and bimodal cues rather than unimodal cues. Populations of sticklebacks from more visually facilitative habitats will respond preferentially to visual cues in situations that induce social behaviors. We also hypothesize that sticklebacks use the olfactory cue in conjunction with visual cues for these affiliations. We predicted that the response to visual

stimuli is greater than the response to olfactory stimuli, but the response to a bimodal stimulus is greater than the response to either a visual or an olfactory stimulus alone. Additional hypotheses about the interactions between trial type and population are not drawn out here, as the means could not be reliably inferred from the analyzed data. Additional trials using the methods described below were performed in an altered pH environment by buffering chemical stimuli and trial tank water to approximately pH 9 through use of Tanganyika Buffer (Seachem Laboratories, Madison, GA). Analysis of this data is in progress, and methods of analysis, results and discussion here refer only to data collected in unbuffered trials.

### Methods

### Animal collection and housing

Fish were collected in minnow traps placed in Bonsall Creek, and Beaver, Brannen, and Weston Lakes in British Columbia, Canada in 2017. Populations were transported to Michigan State University and housed in our lab in separate housing aquaria tanks based on population, with all fish of a population in a single aquarium. Tanks had previously been filled with reverse osmosis water, conditioned with aquarium salt (10 ppm for Bonsall fish, 1 ppm for others), treated with Stresszyme to produce a microbe environment, primer to remove chlorines and ammonias, and cycled to stabilize nitrogen levels Fish were fed a daily *ad libitum* mixture of frozen bloodworms and brine shrimp.

### Experimental set up

Behavioral trials were conducted using three tanks. A trial tank with a 75 x 30 cm footprint was filled to the 25 cm mark with reverse osmosis (RO) water and conditioned with 5 ppm salt, 5 mL of Stresszyme, and tank primer. Two smaller stimulus tanks (30 x15 cm) were placed within the trial tank at each end, which isolates the chemical stimulus. Parallel markings on the exterior of

the trial tank, 15 cm from the center of the trial tank, demarked "association areas" with either stimulus tank from a 15 cm "neutral" area in the middle of the tank (Figure 2.1).

Visual stimulation was provided by placing four members of the focal fish's population in a stimulus tank, while the other stimulus tank was left empty as a visual control. Chemical stimulation was provided by supplying water from the fish population's main housing aquarium through a 0.25 cm polyvinyl tubing to the appropriate side of the trial tank, at approximately 400 mL/min. Water from the trial tank was used as a chemical control. Additional tubing on the back of the trial tank drained water at a rate equal to inflow, maintaining a consistent water level. Tests with dye were used to verify that chemical stimulation was provided by simultaneously presenting visual and chemical stimulation on one side of the tank. Each focal fish was exposed to two types of stimuli (visual, chemical or bimodal) at a time, and received each of three combinations of stimuli ("trial type": visual & olfactory (VO); visual & bimodal (VB); olfactory & bimodal (OB); Figure 2.1A-C). The side of the tank with either stimulus, and the order of trials for each fish were randomized.

After setting up the experimental arena, the focal fish was placed inside a transparent, perforated container in the neutral area, allowing the fish to be exposed to visual and chemical stimulation. The container was removed after a 5-minute habituation period and the fish was filmed for 5 minutes (300 seconds). The amount of time that the fish spent in each area of the of the tank was later quantified by using Jwatcher to analyze the videos. After each trial, all fish were removed and the tanks were drained, sprayed with ethanol, and rinsed in water to remove any residual chemical stimuli.

A fish's attraction for a stimulus in each trial, preference, was used as the response variable. Preference was defined as the difference in the amount of time spent with a specific stimulus for each trial type, and thus could range from -300 to 300, the number of seconds the fish spent in proximity to a stimulus tank. A positive preference score indicates a preference for bimodal stimuli in VB and OB conditions (preference in VB = time with bimodal – time with visual; preference in OB = time with bimodal – time with olfactory) and a preference for visual stimuli in VO conditions (preference in VO = time with visual – time with olfactory). Time spent in between stimuli in each condition was scored as apathy, which is the proportion of time spend in the middle third of the trial tank, i.e., not making a choice.

### **Statistical Analysis**

Statistical analyses were conducted in R. Apathy was used as a covariate for all models testing for measuring preference. Apathy controls variation in how much time was spent with the sensory stimuli, as opposed to between them in the neutral zone, which necessarily decreases preference and could indicate indifference or non-detection of stimuli. Data were fit to linear mixed models with a restriction-estimated maximum likelihood (REML), using the lme function of the package "nlme," to assess the inclusion of random effects by Akaike Information Criterion (AIC). Random effects of individual and nested effects of fish and trial were tested for a specific full model structure containing apathy as a covariate, with trial type and population interaction and main effects. The selected model contained no random intercept. Comparisons of model fixed effects were performed by Likelihood ratio tests. Model selection reduced the full model of the covariate apathy, and fixed effect combinations of population and trial type terms. Models were fit with maximum likelihood and compared via the likelihood ratio test to find the best fitting reduced model.

The selected model, which contained the main effects of population, trial type and apathy, was assessed for the assumptions of a linear model through residual diagnostics. Residuals for these models were not normally distributed, and showed high autocorrelation at lag zero. But as analysis of covariance (ANCOVA) is robust to normality assumptions and we found no better structure of lower level variation, we used type II sums of squares ANCOVA on the models to estimate mean preferences in different trial types. Least square means from the model fit were used to compare differences in preference among trial types and populations with a Tukey adjustment.

## Results

We did find differences among preferences for trial types and populations, though not their interactions. Preference was significantly positive for VO and OB trials, and greater than the preference of VB trials, at the average apathy (Figure 2.2). Among the populations, the fish from Weston and Brannen had preferences for cue types that were significantly greater than preferences demonstrated by Bonsall and Beaver fish populations. Bonsall and Beaver had preferences that were not significantly different from zero (Figure 2.3). Apathy was a significant covariate and increases in apathy decreased preference (Figure 2.2).

### Discussion

While we find that populations differ in vision based preferences for social affiliations, these differences only partially correspond to the expected habitat differences of these populations. We had predicted the pelagic Weston fish and marine Bonsall fish to have higher preferences for visual cues than littoral (Brannen and Beaver) populations, as fish from open water habitats often show relative dominance of vision over olfaction in multiple aspects of their biology (Teichmann 1954; Kasumyan 2004; Atta 2013). But for overall preferences, a mix of populations from

different habitat types, Brannen and Weston, showed significant visual based preferences, greater than that shown by Bonsall and Beaver fish. These results conflict with recent results based on morphology (Chapter 1), where Beaver, Brannen and Weston have similar sensory systems. Yet Beaver behaves more like Bonsall, indifferent to cue types. Brannen and Weston show high preference, which implicates low apathy and/or strong attraction to at least one type of stimulus.

We find no evidence of use of olfaction by sticklebacks in this assay. Associations with visual stimuli were greater than olfactory stimuli alone, as were associations with bimodal stimuli over olfactory stimuli, indicating the prioritization of vision. But that bimodal stimuli did not result in greater associations than visual only stimuli indicates that olfaction is either not used, or is wholly overshadowed by vision in this task. It should be noted that this result is what occurs at the average apathy; at levels of very low apathy the preference for bimodal stimuli over visual stimuli was stronger, which may suggest olfaction is used, but only in extremely reactive, or quick decision making fish. This could suggest information obtained through olfaction alerts a receiver to the presence of senders in the area (Rowe and Guilford 1999; McLennan 2003). Evaluation of this, and the interactions of populations with these treatments to understand how the strength of a visual preference varies among fish of different ecologies are envisioned as future avenues of this research.

APPENDIX

Chapter 2 Figures and Tables



Figure 2. 1 Experimental setup.

Pictorial representation of stimulus presentation in A) VO trials, B) VB trials, C) OB trials. Think lines denote stimulus tank-trial tank border, dashed lines indicated the marking distinguishing neutral from either stimulus zone in a trial. Green represents the dispersion of chemical stimuli, while fish lateral to thick lines indicate fish that provide a visual stimulus. Focal fish is depicted at center. Each focal fish underwent each trial type once.



Figure 2. 2 Treatment and apathy effects on preference.

Change in preference (Time spent with either stimulus in a trial type, see methods for details) in relation to apathy (the proportion of the trial spent not in association with either stimulus) between trial types. Letters at right denote belonging to specific group means (Tukey's adjusted least square means p < 0.05). Dashed vertical line indicates average apathy, were least square means were evaluated. Dashed Horizontal line denotes zero preference.



Figure 2. 3 Population preference over all treatment conditions.

Least-square means of preference for sensory stimuli of different stickleback populations. Error bars denote 95% confidence interval. Positive values indicate and overall positive preference for visual over olfactory, and bimodal over unimodal stimuli. Letters denote belong to specific group means (Tukey's adjusted least square means, p < 0.05).

Sensory				
conditions	Bonsall (28)	Brannen (27)	Beaver (25)	Weston (22)
VO	89.9	112.4	19.7	210.4
VB	-22.8	93.3	-55.3	11.0
OB	0.96	109.8	81.6	171.4

Chapter 03 Olfactory perception of mates in ecologically divergent sticklebacks: population parallels and differences

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#### Abstract

The independent evolution of sympatric species pairs of threespine sticklebacks has provided a natural system to explore how divergent ecologies shape mating preferences. Research has shown that both limnetic and benthic females discriminate against heterospecific males, but not against populations of the same species from different lakes, at least when visual cues are available (Rundle et al. 2000). Olfaction is known to be used in species discrimination by benthic but not limnetic sticklebacks in one of the species-pairs (Rafferty and Boughman 2006), but differences across populations are unknown. We hypothesize that females from benthic habitats make use of olfactory cues to distinguish species, but not lakes of potential mates. We predict limnetic females will not show preferences for males of different species or lakes when limited to only olfactory cues. We exposed gravid females from each benthic and limnetic populations of threespine sticklebacks (Gasterosteus aculeatus) from Paxton and Priest Lakes, British Columbia, to chemical stimuli from nesting males in a Y-maze, recording which stimulus a female chose and how much time was taken to make a decision. We did not find significant differences between female populations in the preference for conspecific over heterospecific male odors. There was also no preference for odors of males from the same or a different lake. In all populations of females, the preference for male odors of different lakes differed between the two species of male odors: benthic male odors from a different lake were selected at a greater proportion than limnetic male odors. The amount of time taken to make a decision differed

between female populations, but only when benthic females ultimately chose a limnetic male odor over a benthic. The preference of conspecific over heterospecific odors, though not strong, may still contribute to reproductive isolation in sympatric sticklebacks, particularly through interactions with other senses and environmental properties.

### Introduction

Perceptual systems evolve under the influence of multiple biotic and abiotic factors of the niche a species occupies (Dangles et al. 2009). Through adaptation, senses may become specialized for particular tasks, as in the detection of conspecific sex pheromones in moths (Schneider 1992), and auditory prey localization in owls (Knudsen and Konishi 1979). The nature of such specializations is particularly apparent when they arise repeatedly in different populations due to parallel evolution. The use of model animals and sensory systems has been critical to obtain detailed understanding of the proximate mechanisms of individual sensory modalities adapted to specific types of environments (Krogh 1929). The focus on specializations in a single modality has in some cases led to the neglect of the contributions of additional modes of perception. However, in ecological contexts, animals may employ a number of senses in order to make behavioral decisions, and focusing on a single specialized modality prevents a complete understanding of behavior (Elias et al. 2005).

The threespine stickleback has long been used to study the importance of specialized sensory modalities. Tinbergen's early work demonstrated the importance of vision to the fish's ecology (Tinbergen 1952), and stickleback vision has remained an active field of research (McDonald and Hawryshyn 1995; Boughman 2001; McLennan 2007; Rick and Bakker 2008; Novales Flamarique et al. 2013). In contrast, the stickleback olfactory system, though demonstrated to have a necessary function in mating behaviors (Segaar et al. 1983), was overlooked for many years, partly in light of anatomical research, which concluded that

sticklebacks had a poor sense of smell (Wootton 1976). This view of the olfactory system would change dramatically in the 21st century, as experimenters began studying the role of olfaction in species recognition and mate choice in multiple members of the Gasterosteidae family (Aeschlimann et al. 2003; McLennan 2003, 2004; Milinski et al. 2005; Rafferty and Boughman 2006; Mehlis et al. 2008; Kozak et al. 2009; Hiermes et al. 2015). These studies have not only demonstrated the importance of a historically de-emphasized sensory modality in these fish, but have also led to new studies on the effects of the ecology of perception and reproductive behavior.

Threespine sticklebacks occupy a number of aquatic systems throughout the northern hemisphere, and have undergone several, and in some cases rapid, adaptive radiations (Wootton 1976; Boughman 2007). In multiple systems, populations have diverged into paired ecotypes such as anadromous/stream, stream/lake, and benthic/limnetic (Hagen 1967; Lavin and McPhail 1985; Thompson et al. 1997; McKinnon and Rundle 2002; Boughman 2007). The benthic-limnetic species pairs have become a focus of speciation research, as the two constitute biological species. Although genetically compatible, benthics and limnetics rarely mate in the wild, and ecological and sexual selection reduces the fitness of hybrids (Vamosi and Schluter 1999; Gow et al. 2007; Hendry et al. 2009). In addition, benthic-limnetic pairs have arisen independently in multiple lakes (Taylor and McPhail 2000), and experimental work has shown that mating preferences hold across lakes: benthics spawn with other benthics from their own or another lake more readily than with any population of limnetics, and limnetics similarly discriminate against benthic populations while accepting limnetics from other populations as mates (Rundle et al. 2000).
It is perhaps not surprising that the behavioral mechanisms of benthic-limnetic isolation have predominantly been studied from the visual perspective, since conspecific recognition has been found to rely heavily on visually mediated traits such as body size and color (Boughman 2001; Boughman et al. 2005). However, increased attention to the role of olfactory systems in the Gasterosteidae has prompted studies that explored the relative importance of olfaction in benthic and limnetic sticklebacks. In Paxton Lake the species have been well studied: benthic females use olfaction to discriminate between species of potential mates (Rafferty and Boughman 2006). Furthermore, benthic females imprint on their father's odor and will show preference for the odor of males they are exposed to shortly after hatching, be they conspecific or heterospecific males (Kozak et al. 2011). Limnetics do not demonstrate such reliance on olfaction (Rafferty and Boughman 2006; Kozak et al. 2011).

These results indicate important differences in the sensory biology of the two species in Paxton Lake. Rafferty and Boughman (2006) suggest that these differences may be a product of the different ecological niches of benthics and limnetics. Bottom dwelling benthics have less access to light due to attenuation and denser vegetation, and feed on organisms that may be hidden or camouflaged by the substrate. The relatively enclosed environment in the structured benthic habitat may make chemical stimuli more readily available and traceable than in open habitats. Limnetics primarily inhabit the open water column where light is more readily available, and they feed on small planktonic organisms that require acute vision to detect. Thus, benthic fish occupy a habitat where visual acuity may be less important, and olfactory cues may have more utility. This may promote the use of olfaction in benthic mate choice, while limnetics' reliance on vision may suppress dependence on olfaction in a perceptual trade-off. Such habitat differences have recently been linked to olfactory

recognition by sticklebacks from lakes with different photic conditions (Hiermes et al. 2015). The intrinsic differences in benthic and limnetic habitats, coupled with the evolutionary independence of benthic-limnetic pairs in different lakes allows for further exploration of how sensory systems evolve and enforce reproductive isolation.

Here we test patterns of mate discrimination across populations of benthic and limnetic sticklebacks when fish only have access to olfactory information. By exploring these behaviors in animals that have evolved in parallel, we ask if selection has resulted in fish from the same type of habitat using olfaction in a similar fashion, as they do vision (Boughman 2001), as an adaptation to their similar ecologies. Females were allowed to choose between odor stimuli of two males: either males of the same species but different lakes, or males of the same lake but different species. We predicted that neither species of female would discriminate between males from different lakes, as long as the two males were of the same species. We further predicted that limnetic females would not show a preference for males of either species because visually biased limnetics should not make substantial use of olfactory cues. However benthic females were expected to show a preference for benthic over limnetic male odor, regardless of lake of origin.

#### Methods

Fish Collection and Housing

Fish were collected from Priest and Paxton Lakes in British Columbia by the use of minnow traps at the beginning of the 2013 and 2014 breeding seasons. Males were identified by the presence of nuptial colors, while females were identified by the appearance of gravidity. Traps were collected, and any animals that were not the target species were released. Species-specific characteristics, specifically, coloration, body size, and shape were used to distinguish between limnetic and benthic species (Boughman et al. 2005). The fish, separated by species,

lake, and sex, were transported to Michigan State University. Once in the laboratory, the fish were housed in aquaria at a density not exceeding 1 fish per 4 liters of water. Fish were fed each morning *ad libitum*. Because the species have distinct feeding styles and diets in the wild that may impact their olfactory signature, benthics and limnetics were given different diets (Bentzen and McPhail 1984). The benthics from each lake were fed only frozen bloodworms. The limnetics from each lake were fed only frozen brine shrimp. As such, our design focuses on the ability of species to recognize olfactory cues, rather than to determine the nature of odor differences between species.

Reproductive males were placed in individual tanks (nesting tanks) and given time and materials to construct a nest. Males were identified as reproductive by their bright red throats, blue eyes, and dark coloration. Tanks for nesting males were equipped with a plastic plant and half of a clay pot (for cover), and a 900 mL tray of sand in which to construct a nest. Males were given grass and *Chara* to use as nesting material. Nesting tanks were cleaned and plant material was replenished every two weeks as part of routine maintenance. The sides of nesting tanks were covered to prevent aggression between neighboring males, and minimize disturbances from the laboratory.

Males were enticed daily to encourage nest building. A gravid female of the same lake and species was placed into a transparent glass jar filled with water and covered with a transparent mesh. The mesh allowed the male and female to receive olfactory and visual cues from each other without coming into physical contact. The jar was placed into a male's tank and left for 10 min while observers noted whether the male had built a nest. Each male was enticed once daily, but females were used two to three times. After every male had been enticed, all tanks were covered, and the females were returned to their home tank.

**Experimental Apparatus** 

Preference tests were conducted in a Y-maze with a main arm of 71 cm, choice arms of 56 cm, and walls 9 cm high. The maze was filled to a depth of 4 cm with reverse osmosis (RO) water. A 38 L tank, elevated 75 cm above the maze, supplied the maze with RO water by gravity through 0.6 cm diameter polyvinyl hoses connected to each choice arm. Two additional hoses at the base of the maze drained the maze, generating a current. The rate of flow from the header to the maze was approximately 1300 mL/min: 650 mL/min into each arm. Containers that supplied stimuli (see below), were elevated above the maze, and connected to each choice arm by gravity-fed hoses. The rate of flow from the stimulus containers was approximately 17 mL/min into each arm. Flow rates were controlled by marked PVC airline control valves connected to each hose. Tests with dye confirmed that flow from each choice arm was laminar and did not mix in the main arm. Between trials the Y-maze and associated materials were cleaned with ethanol and rinsed thoroughly with RO water.

## **Stimulus Preparation**

Chemical stimuli were taken from two different reproductively active males (i.e. those that had built a nest, responded to enticement, and tended their nest on the trial day) for each trial. For each male in the trial, a plastic container was filled with 500 mL of water from the vicinity of the male's nest to use as a stimulus, and the nest itself was placed in the container. We then placed the male that produced the nest into the stimulus container for 10 min in order to add additional odors to the stimulus water. Although a small amount of female odor may have been introduced to nesting tanks during enticements, the brief exposure time, overall volume, and filtration of the tank make it unlikely that female odors influenced the stimulus.

Males were returned to their nesting tanks before trials. For each trial, we used males who built nests within 7 d of each other.

#### **Behavioral Trials**

We performed two types of behavioral experiments. In the species discrimination experiment (n = 61), stimuli used came from males of the same lake but different species (i.e. a Paxton limnetic paired with a Paxton benthic male, or a Priest limnetic paired with a Priest benthic male). In the lake discrimination experiment (n = 57), the two stimuli came from males of the same species but different lakes (i.e. a Paxton limnetic paired with a Priest limnetic male, or a Paxton benthic paired with a Priest benthic male). Due to limitations on the availability of fish from each population, we did not perform tests using males from the same population, or males of different lakes and species.

The side of the maze for each stimulus was randomly assigned in each trial. A ready to spawn female, verified by lightly squeezing the abdomen after trials to ensure the presence of ripe eggs, was used for trials. Females were used at most two times (13 of 106 females) and never in the same experiment (e.g. a female that was used in the lake discrimination experiment could only be re-used in the species discrimination experiment).

The female was placed into the Y-maze once flow in the maze and the stimuli had been started. She was placed into an acclimation area behind a gate at the base of the maze and left undisturbed for 5 min. After 5 min, the gate was slowly raised. During the subsequent 10 min, an observer verified that the fish was exposed to both stimulus streams by her position in the main arm. The choice arm she swam up was recorded, along with how quickly she chose an arm. The trial was considered complete once the fish fully entered either choice arm of the Y-maze, or once 10 min had passed. Trials that were not completed in the time frame, or where the female was not exposed to both stimulus streams, were not included in analyses.

Once the trial was over, the female was tagged with elastomer (Northwest Marine

Technology, Inc., Shaw Island, WA) to allow identification in future experiments, and was weighed, measured, and photographed before being placed back in her tank of origin. The males used were also marked, weighed, measured and photographed at the end of trials. All procedures for handling the fish and their use in experiments were approved by MSU Institutional Animal Care and Use Committee (protocol no. 04/13-092-00).

#### **Statistical Analysis**

Statistical analyses were performed in R version 3.2.1 (R Core Team 2017). We used generalized linear models ("glm") to assess female preference. Preference was scored as a binomial response of females' selection of either conspecific (1) or heterospecific (0) male odor in the species discrimination experiment, and selection of either a male odor from the same (1) or different (0) lake in the lake discrimination experiment. Models used a logit-link function. Female species, lake and the pairing of males in each trial (i.e. if the two male odors were from Paxton Lake or Priest Lake in the species discrimination experiment), as well as all two and three-way interaction terms were used as independent variables. Because very few trials were performed in the first year, year is not included in the models. Tests of significance were based on Chi-square ( $\chi^2$ ) tests, given the binomial nature of our data. An effect size (the odds ratio: the proportional odds of a dichotomous outcome between two groups (Nakagawa and Cuthill 2007)) was determined to estimate the magnitude of differences between female lakes, and female species, for each experiment.

Because there were no differences between lakes, in each experiment the data was pooled across female lakes (that is, we combined benthics from both lakes, and limnetics from both lakes to test the species effect). Binomial tests were used to determine if preferences within a

species of female differed from chance. For species discrimination, a directed test (Rice and Gaines 1994) was used with benthic females, as they were expected to show a preference for conspecifics. For limnetic females in species discrimination, and both species of female in lake discrimination, two-tailed tests were used, as there were no *a priori* expectations of preference.

In addition, a two-tailed binomial test was performed on the individual levels of variables that significantly differed to determine if either level itself differed from chance. This test was only applied to the pairing of males in the lake discrimination experiment.

The amount of time taken to complete trials was our measure of latency. The response variable was square-root transformed to meet assumptions of normality, and fitted to a linear model with female lake, species, and the type of male odor selected, with all two and threeway interaction terms as factors, for analysis in both the species and the lake discrimination experiments. In the event of significant effects, post-hoc comparisons were performed using Tukey's HSD on *a priori* comparisons of biological interest. We performed eight comparisons for the species discrimination experiment: 1) Paxton benthic females vs. Priest benthic females when each chose a conspecific odor; 2) Paxton benthic females vs. Priest benthic females when each chose a heterospecific odor; 3) Paxton benthic females that chose a heterospecific odor vs. Priest benthic females that chose a conspecific odor vs. Priest benthic females that chose a conspecific odor; 4) Priest benthic females that chose a conspecific odor vs. Priest benthic females that chose a conspecific odor vs. Priest benthic females that chose a conspecific odor vs. Priest benthic females that chose a conspecific odor vs. Priest benthic females that chose a conspecific odor vs. Priest benthic females that chose a heterospecific odor vs. Priest limnetic females when each chose a heterospecific odor vs. Priest limnetic females when each chose a heterospecific odor vs. Priest limnetic females when each chose a heterospecific odor; 6) Paxton limnetic females vs. Priest limnetic females when each chose a heterospecific odor; 7) Paxton limnetic females that chose a conspecific odor vs. Paxton benthic

females that chose a limnetic odor; 8) Priest limnetic females that chose a conspecific odor vs. Priest limnetic females that chose a heterospecific odor.

#### Results

Species discrimination was not influenced by whether odors of male species pairs came from Paxton or Priest Lake ("glm", n = 61,  $\chi_1^2 = 0.82$ , p = 0.37). We did not find differences in the selection of conspecific over heterospecific male odors between female lakes, nor female species ("glm", n = 61,  $\chi_1^2$  tests, all p > 0.05). The odds ratio for Paxton: Priest female selection of conspecific over heterospecific male odors was 0.59 (95% CI = 0.20— 1.70).

Although not statistically significant, benthic females selected conspecific over heterospecific odors more frequently than limnetic females. The odds ratio for benthic: limnetic female selection of conspecific over heterospecific male odors was 1.84 (95% CI = 0.66—5.13). Collectively, benthic females selected conspecific over heterospecific odors in 63% of trials, although this did not differ from chance (directed binomial test, n = 30, 95% CI = 0.46—1.00, p = 0.13, Figure 3.1a). Limnetic females selected conspecific over heterospecific odors in 48% of trials, which did not differ from chance (two-tailed binomial test, n = 31, 95% CI = 0.30—0.66, p = 1.00, Figure 3.1a).

In lake discrimination, the selection of male odors from different lakes did not differ between female lake or species origin ("glm", n = 57,  $\chi_1^2$  tests, all p > 0.05, Figure 3.1b). The odds ratio for Paxton: Priest female selection of male odors of the same lake over a different lake was 1.01 (95% CI = 0.35—2.95). The odds ratio for benthic: limnetic female selection of male odors from the same lake over the different lake was 0.90 (95% CI = 0.38— 3.03). Collectively, benthic females selected the male odor from the same lake in 46% of trials, which did not differ from chance (two-tailed binomial test, n = 28, 95% CI = 0.280.66, p = 0.85). Limnetic females selected male odor from the same lake in 45% of trials, which did not differ from chance (two-tailed binomial test, n = 29, 95% CI = 0.27—0.65, p = 0.71).

However, we found the species of male odor did influence selection of lakes: in assessing benthic and limnetic females of both lakes, females selected the benthic male odor from a different lake at a greater proportion than they selected limnetic male odors from a different lake ("glm", n=57,  $\chi_1^2 = 4.32$ , p = 0.03, Figure 3.2). Females selected benthic male odors from their own lake over the other lake in only 31% of trials, a rate marginally different than chance (two-tailed binomial test, n = 26, 95% CI = 0.14—0.51, p = 0.08, Figure 3.2). The limnetic male odors from the same lake were selected in 59% of trials, which did not differ from chance (two-tailed binomial test, n = 31, 95% CI = 0.39–0.75, p = 0.47, Figure 3.2).

The interaction of female species, lake, and the choice of a conspecific or heterospecific male odor significantly affected decision times in species discrimination (three-way interaction,  $F_{1,53}$  = 8.52, p = 0.005, Figure 3.3a). Within each female population, the time to choose benthic or limnetic male odors did not differ (Tukey's HSD, all p > 0.05). Significant differences in latency between females from different lakes occurred only when limnetic male odors were selected. The time Priest benthic females took when they ultimately chose a limnetic male odor was greater than the time taken by Paxton benthic females that chose limnetic odors (Tukey's HSD = 11.17, n = 30, p = 0.009 Figure 3.3a). The difference in latency between Priest and Paxton limnetic females that chose a limnetic male odor approached statistical significance (Tukey's HSD = 8.67, n = 31, p= 0.05, Figure 3.3a). In lake discrimination trials, the time to make a decision did not differ between female species or lakes, nor the lake of the male odor chosen (ANOVA F-tests, all p > 0.05, Figure 3.3b).

#### Discussion

Although we do not find differences between populations in odor discrimination for male species or lake, our results are suggestive that odor may contribute to mate choice. Previous findings indicate that only Paxton benthic females discriminate between benthic and limnetic odor (Rafferty and Boughman 2006; Kozak et al. 2011), and prefer conspecific odors (estimated odds ratio: 13, 95% CI = 0.48—349.52). Our effect size ranged from 0.66 to 5.13, which overlaps with these earlier findings, and trends toward stronger conspecific odor preferences by benthic females. Moreover, when able to use all sensory modalities, Rundle et al. (2000) found the probability of a stickleback spawning with a conspecific to be about 2.5 times the probability of spawning with a heterospecific. Kozak et al. (2009) found similar degrees of conspecific preference reported by Rundle et al., not only in spawning probabilities but measures of female behaviors that precede spawning (estimated odds ratio from Kozak et al. (2009): 1.04, 95% CI = 0.70 - 1.53). It is interesting to note that the preference of both species of females for conspecifics over heterospecifics is more similar when all sensory modalities are available, but is asymmetric when only olfaction is used. Because the probability of heterospecific spawning is low (Rundle et al. 2000; Kozak et al. 2009), even slight differences in preference may contribute to the strong reproductive isolation that exists in these species (Lackey and Boughman 2014).

Olfactory perception is influenced by the local habitat (Heuschele and Candolin 2007; Heuschele et al. 2009), and there is good evidence that the use of olfaction in sticklebacks for discrimination tasks has evolved in response to the environment, and is inherited. Fish bred and reared in common laboratory conditions show population level differences in olfactory use (Hiermes et al. 2015). Furthermore, the population differences Hiermes et al. found appear to correlate to the visual environment; offspring of fish from tea-stained lakes are

more prone to use olfaction than offspring of fish from clear lakes, which indicates that there is selection for olfaction when visual perception is constrained.

This leaves several questions about the interaction of senses and environments in decision making. Chemical cues may interact with other cues to modulate information about the sender (Partan and Marler 1999; Munoz and Blumstein 2012), or be used at particular stages of mating to draw attention to the signaler (Rowe 1999). Thus, chemical communication may interact with known differences between benthic-limnetic pairs in a number of traits, such as body shape, size, color, visual perception (reviewed in McKinnon and Rundle 2002), and the lateral line system (Wark and Peichel 2010).

That all populations of females were more likely to choose benthic male odors than limnetic male odors from a different lake is surprising. One use of olfaction in a number of vertebrates, including sticklebacks, is to evaluate the major histocompatibility complex (MHC) (Aeschlimann et al. 2003; Milinski et al. 2005). The MHC encodes a number of proteins important to immune system functioning; by selecting a mate with an optimal complimentary set of MHC alleles, heterozygote offspring produced may be protected from a broader array of pathogens (Aeschlimann et al. 2003; Milinski et al. 2005). Benthic females may be selecting male odor from the other lake because males from different lakes have more dissimilar MHC alleles. Although differences exist between lakes and species in MHC diversity (Matthews et al. 2010a), additional data on the alleles themselves is needed to see how this would affect interspecific preferences. For limnetic females, selecting a benthic male from a different lake may be a product of reproductive isolation evolving independently in each lake. Reinforcement increases prezygotic mating barriers between hybridizing populations due to selection against heterospecific mating (Servedio and Noor 2003). In

benthic-limnetic pairs this is manifested in sexual and ecological selection against hybrid offspring (Vamosi and Schluter 1999; Gow et al. 2007; Hendry et al. 2009). But the species pairs in Paxton and Priest Lakes have arisen independently of one another (Taylor and McPhail 2000), and discrimination against heterospecifics due to reinforcement is a product of selection only within lakes. Females may be especially likely to reject a heterospecific from their own lake, and discrimination against heterospecifics from other lakes may be but an extension of this bias. Discrimination against heterospecifics within lakes is stronger than that between lakes when fish are able to use multiple types of cues in mate choice (Rundle and Schluter 1998; Rundle et al. 2000). It would be interesting to see if these patterns persist in the absence of olfactory cues.

These results further indicate that while odor is more similar within than between species, populations of species from different lakes still differ in their chemical cues. Odor is comprised of multiple components and derived from a number of ecological factors, and may even be altered due to individual activity and changes over seasons (Sommerfeld et al. 2008). In addition to the MHC complex, populations may differ in the glue (i.e. spiggin) used to make a nest, as there are multiple spiggin alleles and these differ between some populations of sticklebacks (Seear et al. 2015). Dietary differences also influence individuals' olfactory signature and can affect responses in social context (Ward 2004), and might also be used to distinguish species. Furthermore, responses to odor cues can also be plastic, with exposure to common resources altering association preferences in only a few hours (Webster et al. 2007b). As our aim was not to determine the nature of genetic or environmental effects, but to see how females use olfaction during the breeding season, we reflected natural differences between populations by using wild-caught fish sustained on species specific diets, and housed in population specific tanks (Larson

1976). While this would accentuate odor differences between the species, it means we cannot determine the role of diet in odor discrimination.

When choosing between options that are equally preferable, organisms may take longer to make a decision when information is available on each option than when no information is present (Bernays and Wcislo 1994). In both species, Paxton females were quicker to choose a limnetic male odor, which indicates there is a difference in how benthic and limnetic scents are compared in each lake. The quick selection of heterospecific odor by Paxton benthic females may be a speed-accuracy tradeoff (Wickelgren 1977). Priest benthics may be more opposed to the scents of limnetics. As such, Priest benthics spend more time evaluating odors used to discriminate species than do their Paxton counterparts, where the accuracy in choosing a conspecific over a heterospecific odor is impaired by choosing quickly. However, the longest latencies were by Priest benthic females that choose the limnetic odor over the benthic odor, an apparently inaccurate decision, and one which only rarely occurred. Why fish that took longer would make inaccurate decisions is unclear, but learning the neural mechanisms that underlie these decisions may enable their explanation (Chittka et al. 2009).

Olfaction is recognized as an important modality to facilitate speciation in a number of taxa (Smadja and Butlin 2008), and many stickleback populations have olfactory preferences (McLennan 2003, 2004; Hiermes et al. 2015). Although this suggests that the marine ancestor of the benthic-limnetic pairs used olfaction in mate choice, vision may take precedence in light environments that transmit nuptial color, and olfaction may only become important following the invasion of new habitats, which impose new sensory demands and constraints. Has the behavioral incorporation of odor in mating decisions been lost repeatedly in the limnetics, or has it appeared multiple times in the benthics and photically constrained populations? The behavioral

role and evolution of olfactory perception in this system remains a promising avenue for future research.

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APPENDIX

## Chapter 3 Figures and Tables



Figure 3. 1 Olfactory based selection of potential mates by female sticklebacks.

Barplots showing the number of trials in which benthic and limnetic females chose males from (a) different species or (b) different lakes. In choosing between species of male odor, benthic females select conspecific over heterospecific odors more frequently than limnetic females, but the difference is not significant ( $\chi_1^2 = 1.43$ , P = 0.23).



Figure 3. 2 Selection of males of different species, as selected by allopatric or sympatric females based on olfactory cues.

Barplots showing the number of trials in which females from all populations selected a male from their own or the other lake, depending on male species. The proportion of males selected from a different lake is higher when choosing between benthic male odors than when choosing between limnetic male odors ( $\chi_1^2 = 4.41$ , \*P = 0.03). The number of trials in which the benthic male from a different lake is chosen over a benthic from a female's own lake is nearly significant (two-tailed binomial test: probability of success = 0.69, •P = 0.08).

# Parallel evolution in stickleback olfaction



Figure 3. 3 Latency in choosing a potential mate based on olfactory cues by populations of female sticklebacks.

The amount of time for females from each population to select between males of either (a) different species or (b) different lakes. Females from Paxton take less time than females from Priest when choosing a limnetic male over a benthic male. Untransformed means are shown, but analyses used a square-root transformation of the response variable to fit normality assumptions (Tukey's HSD;  $\bullet P = 0.05$ ; \* P = 0.009). Symbols denote means, error bars denote standard error. PaxB = Paxton Benthic female; PrsB = Priest Benthic female; PaxL = Paxton Limnetic female; PrsL = Priest Limnetic female.

Chapter 04 Visual and Olfactory Perception in Response to Altered Humic Environments in Threespine Sticklebacks

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## Abstract

Sensory systems function under the influence of multiple, interacting environmental properties. When environments change, so may perception through one or more sensory systems, as alterations in transmission properties may change how organisms obtain and use information. Humic acids, a natural and anthropogenically-produced class of chemicals, have attributes that may change chemical and visual environments of aquatic animals, potentially with detrimental consequences on their ability to locate necessary resources. Here, we explore how environmental disturbance affects the way threespine sticklebacks (Gasterosteus aculeatus) use visual and olfactory information during foraging. We compared foraging behavior using visual, olfactory, and bimodal (visual and olfactory) information in the presence and absence of humic acids. We found evidence that humic acids reduced olfactory-based food detection. While visual perception was not substantially impaired by humic acids, the visual sense alone did not compensate for the loss of olfactory perception. These findings suggest that a suite of senses still may not be capable of compensating for the loss of information from individual modalities. Thus, senses may react disparately to rapid environmental change, and thereby push species into altered evolutionary trajectories.

Introduction

A single sensory system rarely provides an organism with all information necessary for survival and reproduction. Instead, sensory systems are frequently used in combination (Partan and Marler 1999), particularly in response to changing environmental conditions. Despite that signaling systems frequently work together in nature, sensory systems are often studied separately, which does not accurately represent the full set of sensory information that an individual must process to respond to local ecological conditions (Endler 1992). Because environments can be altered in ways that simultaneously affect multiple senses and signals to different extents, the potential exists for sensory systems to act in compensatory ways in the face of environmental change, such as when olfactory information is prioritized over previouslydominating visual information in the transition from light to dark conditions (Partan 2017; and references within). When evolutionary responses are inadequate, long-term and devastating effects on species and the communities to which they belong may result (Longcore and Rich 2004); thus, it is essential to determine whether and how sensory systems act in compensatory and robust ways.

One such agent of environmental change that has the potential to affect multiple sensory systems simultaneously are humic acids in water bodies. In addition to low level toxic effects of humic substances (Zhao and Zhu 2016), humic acids have the potential to disrupt visual communication by attenuating shortwave and UV light (Morris et al. 1995), resulting in red-shifted or "tea-stained" water. As many animals make use of UV and color signals that are suited to a broad-spectrum habitat, visual detection of these signals may be lost when humic acids are present.

When introduced to a body of water, humic substances are also known to disturb chemical communication in fishes. The impediment to olfaction may occur through several mechanisms. Organic acids may alter the pH of the environment, thereby altering the nature of chemical stimuli by producing involatile salts (Muller-Schwarze 2006b). Humic substances also adsorb hydrophobic molecules such as steroidal pheromones, making them unavailable for chemical communication (Mesquita et al. 2003). Whatever the mechanism, the activity of olfactory receptor neurons can be impeded over a broad range of pheromone concentrations by even minute quantities of humic acids (Hubbard et al. 2002). The detrimental effects of humic acids extend to behaviors as well, demonstrated by the loss of chemically-based mate preferences for conspecifics in swordtails (Fisher et al. 2006) and zebrafish (Fabian et al. 2007) in humic environments.

Given the potential of humic acids to alter these traits, it is perhaps ominous to note that this class of organic acids can arise from both natural plant degradation (Thomas 1997) and synthetic agricultural and gardening supplementation (Geyer et al. 1996). Thus, potential exposure could come through both subtle and predictable events like seasonal plant decay, and substantial, sudden events, like the introduction of agricultural runoff (Geyer et al. 1996; Hansten et al. 1996; Thomas 1997).

We assessed how humic acids modify visual and olfactory cues assessed alone and in combination by threespine stickleback (*Gasterosteus aculeatus*) to determine how visual and olfactory senses respond to altered sensory information. The threespine stickleback has radiated in multiple environments (reviewed in McKinnon and Rundle 2002), which vary in several attributes that may affect the transmission of visual and chemical stimuli (Ormond et al. 2011). As sticklebacks have adapted to these habitats, they have displayed divergence in their reliance

on different sensory systems. For example, limnetic sticklebacks, which occupy pelagic regions, are more sensitive to, and have a greater behavioral preference for, bright nuptial colors than fish from benthic habitats (Boughman 2001). The behavioral and molecular (Rennison et al. 2016) diversification of the stickleback visual system demonstrates how the stickleback sensory system has adapted to the specific habitats in which the fish thrive.

The use of olfaction, although widespread in this fish family (McLennan 2003), also seems to have diverged in many populations. Interestingly, reliance on olfaction tends to be strongest when visual cues are likely reduced: fish from tea-stained environments are able to discriminate populations based on olfactory cues (Hiermes et al. 2015), and benthic sticklebacks which live in more red-shifted habitats (Boughman 2001) have been shown to use olfaction to discriminate con and heterospecific potential mates (Rafferty and Boughman 2006). In contrast, sticklebacks from clear water and limnetic populations have not been shown to use olfaction in these ways (Mobley et al. 2016).

The reliance on different sensory systems has also been shown to be context-dependent across populations of *G. aculeatus*. Increases in turbidity result in increased use of olfactory cues over visual cues during mate choice (Heuschele et al. 2009). Increases in pH also promote female attraction to male olfactory cues (Heuschele and Candolin 2007) and are likely to affect sensation of other cues, such as those used in foraging, in similar ways.

Here we measured sticklebacks' response in the presence or absence of humic acid under three conditions: only visual, only olfactory, or both visual and olfactory (bimodal) information available. We evaluated association with a food stimulus, as well as coughing, an olfactory sampling behavior analogous to sniffing in mammals (Nevitt 1991). We predicted that humic acids would have a detrimental effect on responding to the stimulus through either visual or olfactory perception alone. In this assay, both presence and location of the stimulus were redundantly conveyed through each sensory modality (Partan and Marler 1999), and we predicted the combined use of vision and olfaction would enhance perception, and could thus reduce the deleterious effects of acid. While humic acids have demonstrated consequences for visual and chemical perception in fish (Hubbard et al. 2002; Fisher et al. 2006; Fabian et al. 2007), we add to this knowledge base by exploring how the interaction of the senses affect responses to this substance. By controlling access to visual and olfactory information, we examine how the diverse and complex stickleback sensory system reacts to the potentially multimodal disruptive elements of humic acids and demonstrate how a single ecological perturbance can alter perception of stimuli through different sensory channels in this system.

## Methods

#### Fish Collection and Housing

Our experiments used female benthic sticklebacks collected from Priest Lake, British Columbia, in the spring of 2014. To our knowledge humic acids have not been quantified in this lake, but visual properties of the lake and measures of dissolved organic carbons (Ormond et al. 2011) indicate they are in lower abundance that treatments in experimental trials Fish were fed a mixture of bloodworms (Chironomidae) and brine shrimp (*Artemia*) *ad libitum* daily, but were fasted for 24 hours prior to behavioral trials to increase motivation to respond to a food stimulus (bloodworms) in the experiments.

Fish were housed in 284 or 110 L tanks at approximately equal densities before and between behavioral trials. Two weeks before trials began, we uniquely tagged each fish in the experiment with elastomer (Northwest Marine Technology, Inc., Shaw Island, WA). Fish experienced two trials: one without humic acid, and one with humic acid, in each of three

experimental conditions (see below). Following the first trial, fish were placed back into their housing tank for one week until the second trial was conducted, after which they were housed separately.

## **Experimental Apparatus**

Behavioral trials took place in a 73.5 cm by 30 cm tank, filled to a height of 11 cm with 24.5 L of water. Water flow was generated by a 38 L tank on a shelf 60 cm above the trial tank, which fed water via gravity into the trial tank through two, 6.35 mm diameter polyvinyl hoses. Water flowed out of the tank at the rate of inflow (1250 ml/min) through three hoses attached to the front of the trial tank, maintaining a constant water level. Markings outside the trial tank divided it into lateral regions, demarking a 15-cm neutral zone in the middle of the tank, from the outer regions that possessed containers with (Food Association Zone) or without (Control Association Zone) a food stimulus (Figure 4.1).

We used three types of plastic containers to control the senses that fish could potentially use to perceive the food stimulus. All containers were divided into upper and lower sections by a thin mesh glued to the interior, approximately 5 cm from the bottom of the container. The sides of each container were perforated below the mesh divide. The mesh served as a platform to prevent the food stimulus from escaping the container through the perforations, while still permitting the intended transmission of sensory cues during trials. The container was covered with a lid with three holes: one to allow an aquarium bubbler to generate movement of the food stimulus; one to allow an escape for the excess air supplied by the bubbler; and one by which the hoses from the top tank fed water through the containers into the trial tank. Bimodal (visual and olfactory) trials used clear containers as described above. In trials that only allowed the use of olfactory cues, black containers were used so that the contents of the container could not be seen.

For visual-only trials, a small jar was placed within an unpainted container, to prevent chemical cues from entering the arena. Tests with dye confirmed that water from containers on each side did not mix on opposite ends of the tank, and reached the center of the tank within the acclimation period prior to data collection (described below). Two containers of the same type were used in each trial and placed on opposite ends of the tank, one of which held a food stimulus, while the empty container served as an object control.

#### Food Stimulus Preparation

Each trial used 5 g of bloodworms, a standard food given to the fish in the laboratory, as a stimulus. Frozen bloodworms were thawed prior to the trial and placed on the mesh in one of the containers inside the experimental tank. Bloodworms were rinsed for the visual-only trials because preliminary trials revealed the frozen bloodworms emit red coloration when added to water, which obstructed the view of the bloodworms in the visual-only container. In other trials, water from the top tank washed over the stimulus throughout the trial, and the dye dissipated quickly in the larger tank.

#### **Behavioral Trials**

To test humic acid's effects, thirty fish were randomly divided into one of three sensory treatments: visual only, olfactory only, and visual and olfactory (bimodal) (10 fish per treatment). Trials were paired such that each fish was exposed to a stimulus in the absence and presence of humic acids. For a fish's initial trial, the end of the tank on which the food-bearing container was placed was randomly assigned. Because the mechanistic and long-term effects of humic acids on stickleback sensory systems are unknown, trial order was not randomized; the humic acids exposure always followed the non humic acids exposure.

At the beginning of each trial, the containers and appropriate food contents were placed in the tank and the water flow started before the fish was placed into an acclimation chamber in the neutral zone of the trial tank for 3 min. The acclimation chamber was transparent and allowed water to enter, permitting exposure to the food cues during the acclimation period.

After 3 min, we slowly lifted the acclimation chamber, allowing the fish to swim freely in the tank. For the following 5 min, we used the behavior monitoring software JWatcher to record what tank region the fish was in. Trials were scored as they occurred, as visual glare accentuated by humic acid prevented reliable scoring from video. We also tracked instances of coughing, a chemical sampling behavior in fish (Nevitt 1991). In fish with a non-ciliated olfactory epithelium, such as sticklebacks (Bannister 1965; Honkanen and Ekström 1992),water is drawn across sensory cells via changes in water pressure brought about by changing the volume of the olfactory sac (Nevitt 1991). Characteristic movements of the gill opercula allowed this behavior to be recorded regardless of a fish's orientation. This coughing behavior should therefore provide increased olfactory information about the environment. At the end of the trial, the fish was placed back in its original tank. The experimental tank was cleaned with ethanol and rinsed between trials.

A week later the fish was used in a second trial. These trials included the assigned stimulus treatment and a concentration of 15mg/L of humic acids (TeraVita SP-90, Lancaster, PA). To prevent responses due to familiarization made in the first exposure, the food-bearing container was placed on the end of the tank opposite to its position during that individual's first trial.

An additional group of ten fish served as a control for the effect of repeated exposure to stimuli. These fish, designated the acid control group, experienced the bimodal stimulus

treatment, but were never exposed to humic acids, and instead went through a second bimodal trial, with the food-bearing container on the side opposite to its position in the previous trial.

## Statistical Analysis

For each group of subjects, we analyzed the proportion of the 5-minute monitoring period spent in the food association zone and the number of chemical sampling behaviors ("coughs") observed during trials. Trials were not forced choice, such that subjects that spent all their time in the neutral zone and/or the control association zone were included in analyses, and we tested differences between subjects' first and second trials, resulting in many zeros in the dataset. We initially evaluated food association with a zero-inflated, negative binomial mixed model, but due to the small sample size of each treatment group, and as transformations of the dataset did not improve distributional assumptions of parametric models, we ultimately used nonparametric tests (Wilcoxon Signed Rank Tests) to compare fish in their first and second trials. Tests were performed in R version 3.4.2 (R Core Team 2017). Because of the zeros and resulting ties in the response variables, we used the package "coin", which uses Pratt's method for handling zeroes to provide an asymptotic approximation of the p-value (Pratt 1959). However, as these procedures did not affect statistical significance of results, estimates, confidence intervals, and significance levels reported are those calculated from R's built in "stats" package. Additionally, a Fisher's Exact Test was performed on the number of trials in which coughing did or did not occur in the presence and absence of humic acids.

#### Results

## Food Association

We found that humic acid influenced food associations based on olfactory information. Specifically, when using only olfactory cues, fish were more likely to associate with the food

stimulus when humic acid was absent, but not in the chemical's presence (Wilcoxon Signed Rank Test; V = 6, p = 0.03; Figure 4.2). This was not seen with other sensory treatments. Although fish tested with bimodal cues tended to reduce association with food when humic acids were introduced, this change was modest and not statistically significant (Wilcoxon Signed Rank Test; V = 16.5, p = 0.28) (Figure 4.2).

## Coughing

Coughing, although it occurred infrequently in trials, decreased in the presence of humic acid (Fisher's Exact Test; Odds ratio [95% CI] = 15.42 [3.97, 73.22], p < 0.001; Figure 4.3). When using a single sensory system coughing occurred less often in the presence of humic acids than in their absence (Wilcoxon Signed Rank Tests; for vision: V = 36, p = 0.01; for olfaction: V = 45, p = 0.009). When using both sensory systems, the amount of coughing was the same in humic and non-humic conditions (Wilcoxon Signed Rank Tests; acid-control: V = 27, p = 0.64; bimodal: V = 28, p = 0.55; Figure 4.3).

#### Discussion

Humic acid appears to modify the use of olfactory cues in responding to a food stimulus in this assay. When humic acid was absent and olfactory cues were available, fish associated with the food stimulus more than the control container for olfactory cues alone. Vision used without olfactory cues, however, did not produce a preference for the stimulus. When humic acid was introduced, there was no preference for the food stimulus regardless of the sensory treatment, suggesting that humic acid interferes with detection. These results show an asymmetry, i.e., a greater effect on olfactory cues than visual, in perception by sticklebacks for this task and their response to environmental change. These outcomes align with those found in swordtails, which exhibited reduced association to conspecific chemical cues, but not visual cues, in presence of humic acids (Fisher et al. 2006).

Behaviors that promote sampling of chemical cues are common in many taxa, such as puffing by octopods (Chase and Wells 1986), nose-tapping in urodeles (Jaeger et al. 1986), tongue flicking in reptiles (Cooper 1998), antennal flicking in arthropods (Berg et al. 1992), and coughing in fish (Nevitt 1991). Such behaviors may be initiated by a number of stimuli, including non-chemical sources (Muller-Schwarze 2006b). That we found reduced coughing in the presence of humic acids may be surprising, as fish could be expected to increase sampling under impeded sensory conditions, in order to improve perception of a difficult to detect stimulus. However, increased sampling may only be initiated after detection of the stimulus. If exposure to humic acids precedes or prevents the initial detection of food cues, then efforts to improve cue sampling would not be expected to occur. In addition to the alteration of the sensory environment, humic acids may have detrimental effects to organismal health that modifies an organism's physiology (Zhao and Zhu 2016), and thus behavior (Santonja et al. 2017).

That coughing in fish using bimodal cues was also not significantly affected by humic acids is also surprising. The presence of humic acids may not eliminate detection of either visual or olfactory cues, but instead may reduce detection enough to eliminate the coughing behavior, indicating a "knockdown" rather than a "knockout" of both types of stimuli. If each cue is still available in the bimodal condition, the combined inputs may equal or surpass the threshold needed to engage in this chemical sampling, even in the inhibitory presence of humic acids. Such threshold changes have been demonstrated in parts of the nervous system that receive visual and olfactory information (Dalton et al. 2000).

The nature of the visual-olfactory interaction depends on how stimuli are utilized. When stimuli are temporally or spatially displaced, one modality may serve an alerting function, providing information on the presence of a stimulus, while another modality is used to assess information such as location or quality (Rowe 1999; Rowe and Guilford 1999). In our study, olfaction may have served as an alerting behavior that also prompted the search and localization for food. Without the olfactory cue, the food may never have been noticed in the environment, as found by Webster et al. (2007a); sticklebacks foraging in low turbidity environments foraged equally well in high turbidity, until the chemical environment was manipulated.

Our results indicate vision likely plays a subordinate role to olfaction in this task. In the face of an environmental change, such as the introduction of humic acids, subordinate modalities may be unable to preserve behaviors. While using multimodal cues potentially protects against ecological traps (Madliger 2012; Hale et al. 2015), the way animals process different sensory stimuli may nullify this potential. As in the case of humic acids, other environmental pollutants may affect multiple sensory modalities, adding additional complexity to enduring environmental change (Halfwerk and Slabbekoorn 2015). These types of habitat changes may challenge organisms to acquire additional types of information, such as properties of cues that are not altered by the environmental change, or may promote the rapid adaptation of affected senses, in order to withstand modification of the sensory environment.

Sensory systems serve in multiple behavioral tasks, and multiple environmental factors influence how sensory systems function and evolve (Endler 1992). These connections of sensory abilities evolving to meet different tasks are exemplified by sensory biases for courtship characters that resemble food sources, as in the response of carotenoid pigments by guppies (Rodd et al. 2002). While our results demonstrate that humic acid affects perception in a foraging

context, such manipulations of the environment are likely to carry over into other contexts. Copepod evasion of predators is also reduced in humic environments (Santonja et al. 2017) although this may be due to deteriorated physiological condition brought on by humic substances, in addition to or instead of perceptual impairment. In newts, species recognition and mating preferences are altered by humic acids, apparently due to the alteration of visual (Secondi et al. 2014), but not chemical information (Secondi et al. 2015). Alterations of the light environment in at least one threespine stickleback population have previously been implicated in the collapse of reproductive barriers between sympatric benthic and limnetic populations (Taylor et al. 2006) and collapse of cichlid species (Seehausen et al. 1997). But changes in the visual environment due to turbidity (Engstrom-Ost and Candolin 2007), eutrophication (Seehausen et al. 1997), and dissolved organic acids including tannins (Scott 2001) and humic substances also alter the chemical environment, raising questions of whether these are primarily visual or also olfactory effects on sensory divergence and speciation. These studies highlight that perturbation of the sensory environment can have pervasive damaging effects on adaptation and the maintenance of biodiversity. We need more studies of how organisms integrate information from different sensory modalities in the face of changing sensory environments.

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APPENDIX

Chapter 4 Figures and Tables



Figure 4. 1 Diagrammatic set up of experiment.

Diagrammatic set up of experiment. Cylinders indicate stimulus containers, which contained a mesh (horizontal dashed line) below which the sides of the container were perforated (dots). Black lines in containers indicate presence of food stimulus (bloodworms). Vertical dotted lines show the demarcations that divide the tank into food association, neutral and control regions. Black arrows show water flow from top tank into experimental arena through each stimulus container. Grey lines represent airlines into containers.





Change in the median proportions of time associating with food stimulus for first (no acid present) and second (humic conditions, except in acid control) trials under different sensory conditions. Negative values indicate a reduction in food association in the second trial, relative to the first. Error bars denote 95% ci (wilcoxon signed rank test; \* p < 0.05).



Figure 4. 3 Coughing in humic and non-humic environments.

Number of coughs for first (no acid present) and second (humic conditions, except in acid control) trials under different sensory conditions (wilcoxon signed rank test; \*p < 0.05, \*\*p < 0.01).
## Conclusions

Through these works, we have demonstrated various roles and interactions between visual, chemical, and mechanosensory systems in the radiation of the threespine stickleback. The diversity of sensory interactions of this species may rival, correlate with, and yet differs from other traits that have adapted across the species radiation. Furthermore, these interactions differ in behavioral contexts and environmental conditions; a particular behavior or habitat may be best served by one modality, while another set of environmental conditions may evoke the use of other modalities.

In Chapter 01, morphological measures defined the sensory spaces of stickleback populations. The intersections of sensory systems do not represent the categories of littoral and pelagic for the sensory systems in the same manner as other traits, such as body morphology. Instead we see differential groupings of populations, based on the correlation of eye diameter and olfactory area. This suggests that sensory dimensions of different populations are determined by some as yet unrecognized variables or selective pressures. Conditions experienced during development had no significant effect on sensory trait dimensions, indicating that the variation seen in the wild is a result of genetic sources of variation.

In Chapter 02, vision and olfactory cues are contrasted and combined to measure stickleback social affiliations based on sensory information. The data indicates that sticklebacks, across populations, show preferences towards visual and bimodal stimuli over olfactory cues, but not visual over bimodal stimuli. This supports the longstanding notion that the sticklebacks rely more strongly on their visual system, rather than their olfactory system, to make decisions in this social affiliation task. Although probability inferences could not be made on interaction effects

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between population and sensory conditions, sample data indicates these interactions may exist, and may be as large as either variable alone.

In Chapter 03, we find evidence that olfactory cues are used in isolation among populations to evaluate mate choice to various degrees. Benthic fish are more likely than limnetic fish to choose a conspecific mate based on olfactory cues. Populations may differ within ecotypes; our discovery that Paxton limnetics differ in latency response to the same chemical cues as their benthic counterparts suggests that even limnetics are not anosmic to olfactory stimuli.

In Chapter 04, humic acids are used to investigate immediate response of sensory systems to environmental perturbance. Here we found evidence for olfactory ability serving an important role in foraging, as well as its vulnerability to the disruptive effects of humic acids. These effects are not apparently present when vision can be used in complement. However, behaviors in the presence of humic acids may also have a visual basis, since sampling for chemical cues is reduced when either visual or olfactory stimuli are independently available.

The threespine stickleback, one of the earliest models of animal behavior, has remained a fixture in the field of behavioral ecology, and its sensory systems are a major component in many aspects of its biology. But consideration of the senses in isolation is an incomplete consideration of the environment in which they evolved. Understanding the interactions of these modalities therefore gives us the ability to better control and manipulate many elements of the stickleback's biology in studies of speciation, rapid adaption, cognition, development, conservation, and genetics. Further investigations of behavior aimed at identifying the specific parameters that influence selection on senses is needed, and the stickleback remains an amenable model for such works.

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The sensory drive hypothesis, which for many years has (intentionally or not) fruitfully guided investigations into sensory ecology. The model been modified and refined extensively over the years, as a hypothesis should. As behavioral studies exploring the interactions among senses continues to grow, it will be important to isolate and combine different channels of sensory systems. Adding the interaction among senses as a factor in the evolution of a specific modality will further research about informational needs and integration.

The sensory ecology of a species has been of interest to human beings well before the scientific field began. Stories about how well, or poorly, other beings see, hear, taste, touch or smell fill ancient folklore and modern media. Such stories have resulted in introspection and understanding of how we consider our own environments, and the unique worldviews an individual's senses provide. Yet these unique views are ever changing, defined, and restrained by information that can enter and leave different modalities separately, conditions of the current environment, individual experience, and species evolution. Scientific investigation refines our understanding of the nature and extent of these constraints, providing moments of greater clarity in our long standing efforts to see how others acquire, and assign, meaning in the world around them (Otálora-Luna and Aldana 2017).

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