Ready for a Fight? The Physiological Effects of Detecting an Opponent's Pheromone Cues Prior to a Contest.

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Abstract

Reception of pheromone cues can elicit significant physiological (e.g. steroid hormone levels) changes in the recipient. These pheromone-induced physiological changes have been well documented for male-female interactions, but scarcely in same-sex interactions (male-male and female-female). We sought to address this dearth in the current literature and examine whether mangrove rivulus fish (*Kryptolebias marmoratus*) could detect and, ultimately, mount a physiological response to the pheromone signature of a potential, same-sex competitor. We examined steroid hormone levels in mangrove rivulus exposed to one of three treatments: 1) isolation, 2) exposure to pheromones of a size-matched partner, and 3) pheromone exposure to a size-matched opponent followed by a physical encounter with the opponent. We found that exposure to a competitor's pheromone cues elicited a significant increase in testosterone levels. Increases in testosterone were similar across genetically distinct lineages derived from geographically distinct populations. Further, testosterone levels were similar between individuals only exposed to pheromone cues and individuals exposed to both pheromone cues and a subsequent physical encounter. Our findings led us to generate a number of testable predictions regarding how mangrove rivulus utilize pheromone signals in social interactions, the molecular mechanisms linking social stimuli and hormonal responses, and the possible adaptive benefits of hormonal responsiveness to receiving a potential competitor's pheromone cues.

Keywords: Pheromones, Chemical Cues, Steroid Hormones, Aggression, *Kryptolebias marmoratus*

1. Introduction

Pheromones can have significant influences on the expression of social behaviors (e.g. mate choice; reviewed in [1]). The composition, routes of production and release, and phenotypic (i.e. behavioral and physiological) effects of pheromones have been documented in a number of animal taxa including invertebrates (e.g. crayfish, *Orconectes rusticus*; [2-3]), amphibians (e.g. red-legged salamander, *Plethodon shermani*; [4]), fish (e.g. Mozambique tilapia, *Oreochromis mossambicus*; [5]), and small and large mammals (e.g. Syrian hamsters, *Mesocricetus auratus*; [6]). A significant amount of research has focused on the behavioral effects pheromones have on male-female interactions (reviewed in [1, 7]). A classic example is the male house mouse (*Mus musculus*), which produces and deposits pheromones around its territory that act to both attract and induce receptivity in females [1]. Male terrestrial salamanders (*Plethodon jordani*) release pheromones during courtship, which increase female receptivity and reduce courtship duration [8]. Furthermore, female goldfish (*Carassius auratus*) release pheromones at spawning sites, which signals to nearby males the location of the receptive female [9-11]. Research examining the effects pheromones have on same sex interactions (e.g. male-male contests) has received considerably less attention. Male crayfish produce pheromones during agonistic interactions; pheromone production increases as contest intensity increases, and eventual winners often produce higher quantities of pheromones during agonistic interactions compared to eventual losers [2-3]. At the conclusion of agonistic interactions, dominant male crayfish continue to produce pheromones and claim upstream territories while subordinate males cease pheromone production and are relegated to downstream territories [3]. By doing so, subordinate individuals are constantly exposed to pheromones produced by dominant individuals, which potentially reinforces subordinate status. During agonistic interactions between male Mozambique tilapia,

both opponents frequently urinate and, as a result, release pheromones into the surrounding water [5, 12]. Contest winners and dominant males tend to urinate more frequently, in greater quantities, and with greater potencies compared to eventual losers and subordinate individuals [5]. Pheromone production during these interactions is thought to provide both combatants with information regarding asymmetries in fighting ability between the contestants and often leads to contests settling without the use of physical aggression [12]. Surgically impeding the contestants' abilities to urinate causes increased use of physical aggression during agonistic interactions and protracted fights [12].

Pheromones also can elicit significant physiological responses [4, 7, 13], which may mediate adaptive behavioral changes [14-18]. Pheromone-induced neuroendocrine changes have been well documented in mice and principally for male-female interactions [7]. Female mice exposed to the pheromones of dominant males rapidly enter into and synchronize their estrous cycles (Whitten effect) but exposure to an unfamiliar male's pheromone signature can interrupt this cycle and cause females to abort their current pregnancy (Bruce effect; reviewed in [1, 7]). Exposure to female pheromones elicits significant increases in male androgens (testosterone) and luteinizing hormone, a peptide hormone that regulates spermatogenesis, in male Syrian hamsters [6] and glucocorticoid levels (corticosterone – a stress-related steroid hormone) in male red-legged salamanders [4]. Furthermore, exposure to androstanedione, an androgen and a component of male sweat, elicited a significant elevation in stress hormone (cortisol) levels in human females [13]. Prior investigations have focused heavily on pheromone-induced neuroendocrine responses elicited during male-female interactions but, to the best of our knowledge, there has only been a single study that has investigated pheromone-induced neuroendocrine changes elicited by same sex interactions. O'Connell et al [19] found that when a

male cichlid fish (*Astatotilapia burtoni*) was both visually and chemically exposed to a potential competitor it exhibited a significant increase in androgen levels. We sought to further expand upon this work by examining whether mangrove rivulus fish (*Kryptolebias marmoratus*) could detect and mount a physiological response to the pheromone signature of a potential same-sex competitor.

The mangrove rivulus is a small fish endemic to mangrove ecosystems [20]. Populations of mangrove rivulus can be found as far north as Central Florida, U.S.A to as far South as Venezuela and Rio de Janeiro [20]. Mangrove rivulus are known to be highly aggressive towards conspecifics and have become a model system for investigating aggressive behaviors [21], experience-dependent changes in behavior (e.g. winner and loser effects; [22]) and hormonal modulation of aggressive behaviors [23]. Androgens (testosterone and 11-ketotestosterone) and glucocorticoids (cortisol) in mangrove rivulus change as a function of engaging in aggressive contests (duration and escalation; [23 - 24]). Furthermore, pre-contest androgen and glucocorticoid levels can dictate an individual's probability of initiating, escalating, and winning a contest [23]. Whether mangrove rivulus are responsive (e.g. physiologically) to conspecific pheromones remains unknown. We hypothesized that mangrove rivulus would detect pheromone cues emitted by a conspecific and that they would exhibit significant hormonal changes in response to those cues. We elected to assay two androgens (testosterone and the fish specific 11-ketotestosterone), an estrogen (estradiol), and a glucocorticoid (cortisol) in fish that were exposed, or not, to conspecific pheromones. We chose these steroid hormones based on their connection with aggressive behaviors in the mangrove rivulus (e.g. [23 - 24]) and because prior research has shown that steroid hormone concentrations (e.g. testosterone and cortisol) can change in response to pheromone cues (e.g. [4, 6]). We predicted that pairs of rivulus that are

visually, but not chemically, isolated from one another would exhibit significant increases in hormone levels compared to individuals that receive no chemical cues from a potential adversary. Furthermore, we were interested in whether there was a relationship between pheromone-induced and contest-induced changes in hormones; that is, whether fighting affects hormones more than, less than, or equivalent to olfactory stimulation alone. We predicted that fighting would have an additive effect and that individuals that were both exposed to pheromonal cues and competed in a contest would have higher hormone levels compared to individuals that were only exposed to pheromonal cues.

2. Methods

2.1 Study System

Mangrove rivulus populations are androdioecious; consisting of males and self-fertilizing hermaphrodites [25 - 26]. While there are documented cases of outcrossing between males and hermaphrodites [27], the most common mode of reproduction for rivulus is self-fertilization. Repeated bouts of selfing can result in completely homozygous individuals capable of producing offspring that are genetically identical to parent and all siblings (isogenic lineages; [28 - 29]). Individuals from three distinct, isogenic lineages derived from field-caught individuals collected at various geographic locales (HON9: Utila, Honduras; R2: Roatan, Bay Islands, Honduras; RHL: San Salvador, Bahamas), were utilized in this study. All lineages were confirmed homozygous at 32 highly polymorphic loci [28 – 30]. Only adult hermaphrodites (>6 months of age) were utilized. All focal individuals were housed in ventilated, 1 L Rubbermaid[®] containers filled with 25 ppt salt water (Instant Ocean[®] salt and tap water). All individuals were kept under a 12L:12D photoperiod, a temperature of $26 \pm 1^{\circ}\text{C}$, and were fed daily 2 ml of live brine shrimp

(*Artemia*) nauplii reconstituted in water. The University of Alabama Institutional Animal Care and Use Committee approved all procedures described herein (IACUC #13-309-1).

2.2 Experimental Protocol

All focal individuals were measured (mm) and weighed (g). Average mass of all focal individuals was 0.36 ± 0.08 g (range: 0.2-0.58 g) and average standard length (from snout to caudal peduncle) was 27.38 ± 1.74 mm (range: 23.3-31.8 mm). Afterwards, focal individuals from each lineage were assigned to each of the three treatments based on mass and standard length to keep variance in body size consistent among treatments. In treatment 1, a focal individual was isolated overnight in one compartment of our contest arenas, 31 x 20 x 15 cm Plexiglas containers lined with black corrugated plastic; divided in the center by a black, non-reflective partition. This treatment was used to quantify hormone concentrations of individuals that were not exposed chemically, visually or physically to a potential competitor (N=24; 8 per lineage). In treatment 2, a focal individual and a communal partner were housed overnight in the right and left compartments of the contest arena, respectively. The focal individuals and communal partners were matched by lineage and size ($\leq 10\%$ mass asymmetry). The focal individual and communal partner were visually, but not chemically, isolated by an opaque black partition. This treatment was used to quantify hormone concentrations of individuals that were chemically, but not visually or physically, exposed to a potential competitor (N=24; 8 per lineage). It was confirmed that the partition allowed water to pass between compartments in a pilot study; non-experimental fish, one in each compartment, were placed into the contest arenas, food dye was added to the right-hand side compartment, and at 0800 h the following day, it was found that the dye had been evenly dispersed throughout both compartments. In treatment 3, two

focal individuals, matched by lineage and size ($\leq 10\%$ mass asymmetry), were housed overnight in the contest arenas separated by an opaque partition. At 1000 h the following day the partition was removed and the individuals allowed to interact until the contest settled (first retreat). Following settlement, the individuals remained in the fight tanks for an additional three minutes to further confirm contest status (win or loss) of both individuals and that reversal of status did not occur (no reversal occurred). Contests were digitally recorded and a research assistant blind to the hypotheses being tested quantified behaviors using the JWatcher behavioral quantification program (<http://www.jwatcher.ucla.edu/>). Behaviors quantified were: 1) latency to resume normal swimming behavior (recover) following disturbance (partition being lifted); 2) identity of the individual that first recovered; 3) latency to initiate (approaching or threat displaying at opponent); 4) identity of individual that initiated the contest; 5) number of approaches; 6) number of threat displays; 7) number of bites; 8) number of circle bouts (synchronous attack where both competitors chase each other in a circular motion) ; 9) number of mouthlocks; 10) number of avoids (slow movement away from opponent); 11) number of retreats (rapid movement away from opponent); 12) status (winner or loser); and 13) contest duration (time between initiation and first retreat). In treatment 3, focal individuals were exposed to a competitor chemically, but not visually, prior to a contest and then were given the opportunity to engage physically with the competitor (N=48; 16 per lineage). Once the behavioral assays were completed, hormones were collected from all individuals. Focal individuals from treatment 1 and 2 remained in their respective fight tanks for the same duration of time as focal individuals from treatment 3.

2.3 Hormone Collection, Extraction and Analysis

Steroid hormones were obtained using a non-invasive, water-borne collection technique, which has been validated for use in mangrove rivulus [23]. Hormone collections were performed on all focal individuals from all treatments (1, 2, and 3) simultaneously. Individuals were transferred from the fight tanks to beakers filled with 500 ml of 25 ppt salt water for 1 h, during which time steroid hormones passively diffused across the gills and into the surrounding water [31]. The water samples were then filtered using Whatman filter paper (Grade 1) and then pulled through Waters, Inc. Sep-Pak C-18 extraction columns (500 mg, 3cc; primed with 2 x 2 ml methanol and 2 x 2ml distilled water) using a vacuum manifold. Excess salts were removed from the columns by drawing 2 x 2 ml of distilled water through the column after the water samples containing hormone had been pulled through. Columns were stored at -20°C until assays were performed. Prior to assays, the columns were thawed and 2 ml of distilled water purged any remaining salts. Steroid hormones were eluted by passing 2 x 2 ml ethyl acetate through each column into 13 x 100 mm borosilicate vials. Eluted samples were placed in a 37°C water bath and dried with a gentle stream of ultra-pure nitrogen gas using an evaporating manifold. Samples were resuspended in 30µL of 100% ethanol and vortexed for 1 min. Enzyme-immunoassay (EIA) buffer provided with the Cayman Chemicals Inc. kits (570µL) was added to each sample, and vortexed for 5 min. Serial dilutions were performed on pooled samples – extracted (45 µl per sample) from all resuspended samples – to determine the appropriate dilutions needed to properly assay each hormone. Cortisol, 11-ketotestosterone, and estradiol were assayed at a 1:1 dilution while testosterone was assayed at a 1:4 dilution (50 µl of original resuspension in 150µl EIA buffer). Values reported in results have been corrected for dilutions. Instructions provided by Cayman Chemicals, Inc. were followed strictly. Samples were run on 3, 96-well EIA plates,

with control samples at the beginning and end of each assay plate; controls were pooled samples. Intra-assay coefficients of variation were (plates 1 through 3) 3.65%, 1.16%, and 1.92% for testosterone; 9.65%, 0.78%, and 1.24% for 11-ketotestosterone; 1.80%, 3.14%, and 1.36% for estradiol; 1.13%, 1.19%, and 6.79% for CORT. Inter-assay coefficients of variation for testosterone, 11-ketotestosterone, estradiol, and cortisol were 2.90%, 5.70%, 2.38%, 9.80%, respectively. Analyses on the relationship between the standard and serial dilutions curves for all hormones [32] were performed, which revealed that parallelism between the standard and serial dilution curves had been achieved (testosterone: $t_{12}=0.16$, $p=0.876$; 11-ketotestosterone: $t_{11}=2.089$, $p=0.067$; estradiol: $t_{10}=0.104$, $p=0.919$; cortisol: $t_{11}=0.075$, $p=0.941$).

2.4 Statistical Analyses

All statistical analyses were performed using JMP (v. 7.0.1 SAS Institute Inc., Cary, NC, U.S.A.). The effects of treatment (1, 2, and 3), lineage (HON9, R2, and RHL) and the treatment x lineage interaction on steroid hormone concentrations were determined using analyses of covariance (ANCOVA). Fish mass (g) was included as a covariate to account for variation in hormone levels due to variation in individual mass; that is, larger individuals may release more hormones due to larger body size [31]. Hormone concentrations were natural log transformed to achieve normality. Tukey's Honest Significant Difference (HSD) multiple comparison tests were performed to evaluate differences among levels of the main effects and interactions. For treatment 3 only, a principal components analysis (PCA) was used to distill all aggressive behaviors down to two principal components. Only principal components with an eigenvalue ≥ 1 were retained. Mouthlock behavior was excluded from the PCA because the behavior was represented in very few contests (4 of 48). The effects of contest status (win or loss), contest

initiation (discrete variable; yes or no), and contest performance (i.e. principal components) on steroid hormone concentrations were analyzed using a repeated measures analysis of covariance (RM-ANCOVA) with status and initiation as the repeated measure and fish mass as a covariate. This approach is considered more powerful relative to a traditional ANOVA and helps account for non-independence between subjects (e.g. winners and losers; [33]).

3. Results

We found significant differences in hormone concentrations among-treatments (Table 1). Testosterone concentrations were significantly lower in isolated individuals (treatment 1) compared to individuals exposed to a potential competitor's pheromones alone (treatments 2) and individuals exposed to a potential competitor's pheromones followed by a fight (treatment 3; Fig. 1). Concentrations of 11-ketotestosterone, estradiol, and cortisol did not significantly differ among treatments (Table 2). We also found significant differences in hormone concentrations among lineages (Table 1 & 2). RHL had significantly lower concentrations of 11-ketotestosterone, estradiol, and cortisol compared to HON9 and R2 (Fig. 2). HON9 had higher testosterone concentrations compared to R2 and RHL (Fig. 2). All interaction effects were non-significant, indicating that the effects of treatment and lineage were independent of one another (Table 1).

From the PCA, principal component 1 (PC1) explained 34.22% of the behavioral variation and loaded heavily on submissiveness: high numbers of avoids and retreats. Principal component 2 (PC2) explained 20.65% of the behavioral variation and loaded heavily on aggressiveness: high numbers of approaches, threat displays, and bites. For treatment 3, we found that neither initiating the contest (yes or no) nor did contest status (win or loss) correlate in

significant ways with post-fight steroid hormone concentrations (Table 2). We did not find any significant correlations between behaviors performed (PC1 and PC2) and post-fight steroid hormone concentrations (all $p > 0.05$).

Table 1 – *Summary of Hormone Analyses*. Effects of treatment (1, 2, and 3), lineage (HON9, R2, and RHL), mass, and their interactions on mean-level steroid hormone concentrations. Significant effects ($p < 0.05$) are shown in bold.

	Testosterone		11-Ketotestosterone		Estradiol		Cortisol	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Model (overall)	3.34	0.0001	1.89	0.032	2.55	0.0029	2.11	0.014
Treatment	6.42	0.0026	1.69	0.19	0.34	0.71	2.09	0.13
Lineage	12.77	<0.0001	8.41	0.0005	9.49	0.0002	8.49	0.0005
Mass	0.20	0.66	1.25	0.27	0.40	0.53	0.41	0.52
Treatment x Lineage	1.85	0.13	1.21	0.31	0.41	0.80	0.17	0.95
Treatment x Mass	0.25	0.78	2.05	0.14	0.22	0.80	0.17	0.85
Lineage x Mass	0.90	0.41	1.01	0.37	0.27	0.76	0.24	0.79
Treatment x Lineage x Mass	1.29	0.28	0.60	0.66	1.08	0.37	0.74	0.57

Table 2 – *Summary of Repeated Measure ANCOVA*. Due to the non-independence between winners and loser in treatment 3 a separate analysis was conducted to examine the effects of lineage (HON9, R2, and RHL), status (win or loss), initiation (yes or no), size, contest dynamics (e.g. duration) and their interactions on mean-level steroid hormone concentrations. Significant effects ($p < 0.05$) are shown in bold.

	Testosterone		11-Ketotestosterone		Estradiol		Cortisol	
	F-value	p-Value	F-value	p-Value	F-value	p-Value	F-value	p-Value
Lineage	9.18	0.0009	4.41	0.02	4.92	0.01	5.81	0.008
Status	2.03	0.17	1.14	0.30	1.59	0.22	0.98	0.33
Length	0.08	0.78	0.01	0.99	0.53	0.47	0.07	0.80
Aggressiveness	0.10	0.77	0.15	0.70	0.01	0.99	0.70	0.41
Submissiveness	0.76	0.39	0.76	0.39	1.53	0.23	0.01	0.99
Duration	0.47	0.50	0.39	0.54	0.01	0.96	0.10	0.76
Status x Length	2.67	0.11	1.42	0.24	1.99	0.17	1.44	0.24
Aggression x Status	0.05	0.82	0.01	0.92	1.90	0.19	0.01	0.99
Submission x Status	0.46	0.50	0.52	0.48	0.60	0.45	0.11	0.74
Duration x Status	2.91	0.10	1.21	0.28	0.70	0.41	0.82	0.38

Figure1 – *Comparison of Testosterone Concentrations Among-Treatments*. Testosterone concentrations reported as natural log transformed. Isolate (treatment 1) is represented by the white bar, communal (treatment 2) is represented by light gray bar, and communal + fight (treatment 3) is represented by the dark gray bar. Treatments separated by different letters are considered significantly different.

Figure 2 – *Comparison of Steroid Hormone Concentrations Among-Lineages*. Hormone concentrations are reported as natural log transformed. HON9 is represented by the white bar, R2 is represented by light gray bar, and RHL is represented by the dark gray bar. * denotes significance.

Figure 1

Figure 2

4. Discussion

Exposure to pheromones elicits significant neuroendocrine responses during male-female interactions [4, 6, 7, 13]. However, whether or not pheromone signals produced and received before or during same-sex interactions (e.g. male-male or female-female) also elicit significant neuroendocrine responses has received far less attention (but see [19]). Here we investigated whether mangrove rivulus could detect and mount a neuroendocrine response to the pheromone cues from same-sex individuals. We found that androgen levels, specifically testosterone, were elevated in individuals exposed to the pheromone cues of a potential competitor relative to unexposed individuals. These neuroendocrine changes were similar across genetically distinct lineages derived from geographically distinct populations. Further, elevation in androgen levels was similar between pheromone-exposed individuals that did not engage in a subsequent contest and those individuals that did engage in a subsequent contest. Our findings support our hypothesis that mangrove rivulus can detect the pheromone cues of potential competitors and that reception of these cues elicit significant changes in neuroendocrine state. Utilizing our findings we have generated a number of testable predictions on: 1) how mangrove rivulus utilize pheromones before and/or during social interactions, 2) the potential mechanisms, e.g. neural, mediating pheromone reception and subsequent neuroendocrine responsiveness, and 3) the potential adaptive benefits of this hormonal responsiveness to a competitor's pheromone cues.

It has been established that mangrove rivulus gather and utilize information about their opponents to alter their decisions during aggressive contests. For example, mangrove rivulus utilize prior contest experiences (status: win or loss; dynamics: escalated or not) to reassess their fighting ability in subsequent contests [22, 34-36]. It also has been discovered that mangrove rivulus can assess an opponent's fighting ability during a contest and gauge whether or not to

persist (mutual assessment; [37]). However, the routes through which mangrove rivulus gather this information (e.g. physical, visual, and/or chemical) have yet to be determined. As seen in other aquatic animals such as crayfish and tilapia, pheromone cues can be a significant source of information on: 1) an opponent's motivation to compete, and 2) whether an individual should persist or retreat from the contest [3, 12]. Surgically debilitating the release of pheromone cues leads to prolonged contests that escalate more frequently compared to contests where combatants are able to utilize pheromone cues [12]. Our findings indicate that mangrove rivulus are receptive to pheromone cues produced by a potential competitor and that they exhibit neuroendocrine responsiveness to those cues. We predict that, during a contest, mangrove rivulus can utilize pheromone signals among other sources of information produced by a competitor to alter their competitive decisions (e.g. persist or retreat) before, during, and/or after the interaction.

Immediate early gene (IEG) expression is often considered the first component of a molecular cascade that mediates an individual's physiological and, ultimately, behavioral responsiveness to a social stimulus (i.e. ascension to dominance; [38]). In fish, pheromone reception can induce significant changes in neural activity through increased IEG expression within brain regions known to regulate social behaviors [19, 39 – 42]. In male goldfish, reception of female pheromones elicits significant changes in c-fos expression within the pre-optic area; specifically in neurons rich with androgen receptors [42]. Additionally in male goldfish, reception of male pheromones elicited significant increases in arginine vasotocin (AVT), a nonapeptide known to mediate social behaviors, gene expression within the pre-optic area [41]. In male cichlid fish, reception of visual and/or pheromone signals from a potential competitor differentially elicited significant changes in c-fos expression in dopaminergic neurons within the

central region of the ventral telencephalon (a homolog to the mammalian striatum; [19]) This change in c-fos expression correlated with significant elevation in androgen levels and increased aggressiveness. Changes in IEG expression can potentially play two, non-exclusive roles: 1) act as a trigger that starts the physiological cascade leading to changes in physiology (e.g. hormone production) and behavior or 2) prepare the brain for activation by an influx of hormones produced following a social stimuli [38]. We predict that, following the reception of a competitor's pheromone cues, mangrove rivulus exhibit similar changes in gene expression within brain regions associated with social behaviors. These changes in brain gene expression may then either elicit the elevation in androgen levels observed in our study and/or prepare the mangrove rivulus's brain for the influx of androgens following pheromone reception.

Androgens are a well-known mediator of aggressive behavior [23, 43 – 44]. Further, an individual's androgen levels preceding an agonistic encounter are known to have significant effects on their aggressiveness during a contest and their probability of victory [23]. It has been hypothesized that individuals may anticipate an impending agonistic interaction by cueing in on the visual, auditory, and/or chemical signals intentionally or inadvertently produced by a potential opponent [14-18]. The adaptive value of anticipating an ensuing agonistic encounter is that an individual may elevate their aggressiveness prior to engaging in the contest, which could increase that individual's probability of winning the interaction – the so-called “Primer Effect” [14 - 15, 18]. It also was hypothesized and later supported that underlying this increase in aggressiveness were anticipatory changes in the individual's neuroendocrine state elicited by receiving cues (i.e. visual) prior to the contest that indicated imminent interaction [15, 18]. Our results demonstrate that mangrove rivulus can exhibit an anticipatory neuroendocrine response to a potential competitor's chemical cues. Whether the changes in androgen levels observed

correlate with changes in aggressive behaviors (i.e. a true primer effect) could not be definitively determined by our experimental design because both combatants in treatment 3 were exposed to pheromone cues. Nonetheless, we predict that elevation in androgen levels prior to a contest may be a mechanism underling a possible primer effect in the mangrove rivulus.

There were significant lineage differences in neuroendocrine state. These results are consistent with previous work in mangrove rivulus [23 – 24, 35]. We also found that these lineage differences were independent of treatment differences; that is, there were no significant treatment x lineage interactions (Table 1). The lack of treatment x lineage interactions indicates that all lineages are responding to pheromone cues in a similar fashion. This suggests that the observed neuroendocrine changes may have some adaptive value and thus are conserved across lineages derived from geographically distinct populations. Whether and how these pheromone-induced neuroendocrine changes are adaptive to mangrove rivulus populations have yet to be determined. It has been hypothesized that being “primed” for an imminent contest has both short- and long-term benefits [14 - 15]. The short-term benefit is that primed individuals tend to fight harder and have an increased probability of winning their contest (primer effect; [14]) and the long-term benefit is that individuals that win current contests have a higher likelihood of winning future contests (winner effect; [22]). The long-term benefits are applicable to the mangrove rivulus system while the short-term benefits require further investigation. First, mangrove rivulus are known to respond to prior winning experiences with increased propensities to escalate and winning future contests (winner effects; [22, 35-36]. Second, as we have demonstrated in this study, rivulus exposed to pheromone cues of potential competitors had higher testosterone levels relative to unexposed individuals. We also found that individuals exposed chemically, but not visually or physically, to a potential competitor (treatment 2) had

steroid hormone levels equal to those of individuals that were chemically, but not visually, exposed to a potential competitor and then faced that competitor in a subsequent fight (treatment 3; Fig. 1). Pre-fight androgen concentrations can have significant effects on mangrove rivulus's contest performance [23]. In paired contests, the eventual winner that had higher pre-fight testosterone concentrations tended to deliver more attacks against the eventual loser [23]. It is possible, but yet to be determined, that elevations in pre-fight hormone levels would precipitate increased aggressiveness and, consequently, the individuals' probability of winning the contest. Nonetheless, if the response to chemical cues is under strong positive selection, then the expectation is that the response should be pervasive and similar among lineages. Our findings demonstrate multiple mangrove rivulus lineages possess equivalent physiological responsiveness to pheromone cues and lead us to suspect that responsiveness to pheromone cues has some form of adaptive value within the mangrove rivulus system.

Pheromone-induced neuroendocrine changes have the potential to not only influence pairwise interactions but also larger social networks. Visual, auditory, and chemical signals often are produced with the intention of conveying information to an intended receiver. However, bystanders within range of the signal can also receive this information and modulate whether and how they interact with the signal producer(s) (i.e. social eavesdropping; [45 – 47]). Green swordtail fish (*Xiphophorus helleri*) bystanders that witnessed a contest between pairs of male conspecifics alter how they interact with the observed combatants – bystanders tend to avoid prior winners and prior losers that escalated the contest [45]. Male fighting fish, *Betta splendens*, that observe agonistic interactions between pairs of male conspecifics increase their aggressive motivation and tend to attack all opponents, not just the individuals they observed, more aggressively than naïve males [16]. Furthermore, female crayfish (*Procambarus clarkii*) that

were visually and chemically exposed to agonistic interactions between males associated more often with the winner compared to the loser [46]. The mechanism(s) underlying the behavioral decisions of eavesdroppers, however, have yet to be fully elucidated. Mozambique tilapia bystanders observing an agonistic interaction had higher androgen levels (testosterone and 11-ketotestosterone) relative to naïve individuals [17]. It was postulated that these elevations in androgens could mediate the behavioral changes elicited by eavesdropping on future agonistic interactions [17]. Our findings suggest that pheromones, separately or in combination with additional stimuli, may alter an eavesdropper's neuroendocrine state, which could potentially alter their behavioral decisions in subsequent interactions.

We did not find any significant correlations between contest status, contest initiation, behaviors performed, and steroid hormone concentrations (Table 2). Steroid hormones such as testosterone and cortisol are known to change rapidly following a contest for both winners and loser [49 - 51]. Differences in hormone profiles between winners and losers often appear only after prolonged cohabitation of the two combatants (e.g., [51]). We only provided a three-minute period of cohabitation following contest settlement, which was likely insufficient time to reinforce contest status and for differences in steroid hormone concentrations to appear. Steroid hormones also are known to change as a result of contest dynamics (e.g. escalated vs. non-escalated). In green anole lizards (*Anolis carolinensis*), individuals that performed more pre-settlement aggression (i.e. approaches) had higher testosterone levels while individuals that performed more post-settlement submission (i.e. avoids and retreats) had higher cortisol levels [53]. Furthermore, in Arctic charr (*Salvelinus alpinus*), both contest winners and losers that performed more aggressive acts during a contest had elevated androgen levels (testosterone and 11-ketotestosterone; [54]). Based on our findings, contest dynamics appeared to have had little or

no bearing on post-fight steroid hormone concentrations in mangrove rivulus. This is contradictory to previous work in rivulus, which showed elevated post-fight testosterone, 11-ketotestosterone, and cortisol in losers that engaged in escalated contests compared to losers that engaged in non-escalated contests ([23]; see also [24]). Furthermore, losers that received more post-settlement attacks had decreased testosterone levels while winners that performed more post-settlement attacks had lower cortisol levels [23]. Differences between the findings of the current study and previous works may be related to differences in isogenic lineages utilized. This study utilized the HON9 and RHL lineages, which Earley and Hsu [23] and Earley et al. [24] also utilized. However, Earley and Hsu [23] and Earley et al. [24] utilized two (SLC8E: St. Lucie County, Florida, USA; VOL: Daytona Beach, Florida, USA) and three (DAN2K: Dangria, Belize; SLC8E: St. Lucie County, Florida, USA; VOL: Daytona Beach, Florida, USA) lineages, respectively, that were not utilized in this current study. As stated earlier, rivulus lineages are known to vary in their steroid hormone profiles [23 – 24, 35]. It is thus possible that correlations between post-fight hormone levels and contest performance are lineage specific.

Pheromones can have significant impacts on an individual's behavior and physiology [1, 7, 12]. Numerous lines of research have focused on how pheromones mediate complex social interactions between males and females (reviewed in [1]) but less has been done to understand how pheromones mediate same sex interactions. Pheromones produced during agonistic interactions can communicate both combatants' fighting prowess and motivations, and can lead to faster contest settlement [12]. Few experiments have investigated whether reception of pheromones prior to a contest might influence an individual's behavior and/or physiology [14]. In this study we found that mangrove rivulus detect pheromone cues and that exposure to these cues caused a significant increase in androgen concentrations. Our findings have led us to

generate a number of testable predictions on: 1) the neural mechanisms underlying a mangrove rivulus's neuroendocrine responsiveness to pheromone cues and 2) the potential adaptive benefits, e.g. behavioral, to the observed neuroendocrine responsiveness.

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