

Sex Recognition via Chemical Cues in the Sex-Role-Reversed Gulf Pipefish (*Syngnathus scovelli*)

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Abstract

Sexual selection theory predicts that members of the choosy sex, usually females, should employ multiple sensory systems to obtain information about potential mates. Such predictions should also apply to systems in which sexual selection acts most strongly on females (i.e. sex-role-reversed species), and males of these taxa should be the individuals employing multiple cues to assess female attractiveness. Very little work has been directed toward mate choice involving multiple sensory systems in sex-role-reversed taxa, but fishes of the family Syngnathidae (pipefishes, seahorses, and sea dragons) provide an excellent opportunity to contribute to this research enterprise. While much is known about visual communication in pipefish, the role of chemical communication has not been investigated. Using dichotomous choice tests, we found that male, but not female, Gulf pipefish attended to chemical cues of opposite sex conspecifics. Given that males distinguished sex on the basis of chemical cues, we also tested whether males could assess female body size, an important trait with respect to mate choice in pipefish, on the basis of chemical cues alone. When given the choice between chemical cues produced by large vs. small females, males exhibited no preference. Our results suggest that male pipefish can use chemical cues to distinguish between males and females but not to differentiate females of different body size.

Introduction

Mate choice is often based on multivariate suites of traits perceived in more than one sensory modality (Partan & Marler 2005). A variety of scenarios have recently been proposed to address how these complex sets of traits evolve (Candolin 2003; Hebets & Papaj 2005) and interact to elicit receiver responses (Rowe 1999; Partan & Marler 2005; Chenoweth & Blows 2006). Sex-role-reversed systems provide an excellent opportunity to examine complex trait evolution because the same processes underlying evolution when males are experiencing stronger sexual selection should apply when females experience

stronger sexual selection, except that the constraints on the sexes differ in sex-role-reversed taxa compared to taxa with ordinary sex roles. For example, in sex-role-reversed species females must evolve traits involved in competition for access to mates, while still producing energetically expensive gametes. In addition, male choice has evolved recently in most sex-role-reversed taxa, so the history of evolution of multivariate signaling may be better reconstructed in such taxa. The first step is to characterize the sensory modalities involved in mate choice in sex-role-reversed species.

Over 20 years of research has been conducted on mate choice in pipefishes and seahorses (Berglund

et al. 1986; Vincent & Sadler 1995; Vincent et al. 1995; Billing et al. 2007), but information regarding the use of sensory modalities other than vision is scant (Fish 1953). Syngnathids are characterized by an extreme form of male parental care in which a female's eggs are transferred to a specialized epithelium on the male's ventral surface. Some syngnathids exhibit sex-role reversal which occurs when males are more of a limiting resource in reproduction than females (Berglund et al. 1989; Vincent et al. 1992; Wilson et al. 2003) and therefore sexual selection is more intense in females (Jones et al. 1999). As predicted by sexual selection theory, the females of sex-role-reversed syngnathid species are often more ornate than males and compete with other females for access to males (Trivers 1972; Williams 1975; Andersson 1994). The Gulf pipefish, *Syngnathus scovelli*, is a sex-role-reversed species of pipefish with a range from the north-eastern Gulf of Mexico to northern Brazil. This species displays numerous sexually dimorphic behavioral and morphological traits. Females are larger than males in body length, depth, and weight. Females are ornamented (iridescent blue bands running dorso-ventrally on the trunk) while males are cryptic. Both males and females practice vertical swimming during courtship, but only females exhibit courtship posing by erecting their dorsal fin, darkening their skin around the iridescent bands, posturing in an S-shape, and erecting their darkened ovipositor (Jones et al. 2001; pers. obs. N. Ratterman, A. Jones). Parentage analysis of *S. scovelli* has revealed strong sexual selection on female body size and ornamentation, and no observed sexual selection on males (Jones et al. 2001). The Gulf pipefish thus provides an excellent opportunity to investigate signaling dynamics when sexual selection acts more strongly on females than on males.

The goal of this study was to evaluate the use of chemical cues in mate choice in the Gulf pipefish. Because there appears to be no clear pattern with respect to which sex is most likely to release chemical cues (Liley 1982; Müller-Schwarze 2006), we investigated the response of both sexes to putative cues collected from same and opposite sex conspecifics. We were also interested in the hypothesis that males could detect body size differences in females based on chemical cues alone (females were not tested for size discrimination because our first experiment revealed that females fail to respond to chemical cues of both sexes). We expected males to attend to the chemical cues of large over small females, because males of some pipefish species (including *S. scovelli*) have been

shown to prefer large females (Berglund et al. 1986; Rosenqvist 1990; Jones et al. 2001; Silva et al. 2007). We were thus asking two questions in our study: (1) Do male and female pipefish use chemical cues in sex recognition? and (2) Are males able to assess female body size using only chemical cues?

Methods

Study Animals

Gulf pipefish were collected with a beach seine (2 m × 1 m, 0.1 mm mesh) from shallow seagrass beds in the Gulf of Mexico near Aransas Pass and Port Aransas, TX, USA between May and Sept., 2007. Fish were returned to Texas A&M University in College Station, TX and housed in 21-l glass aquaria (40 × 20 × 25 cm). Fish were supplied with live *Artemia* nauplii *ad libitum* twice daily. Temperature ranged from 30 to 31°C, mimicking natural conditions during the period of time over which the experiment was conducted. Photoperiod was a 15:9 h L:D cycle. Tanks were supplied with aerated artificial seawater (26‰). Only sexually mature individuals were used. Male sexual maturity was diagnosed by the presence of a fully developed brood pouch, and females were considered sexually mature when ovaries extended the length of the trunk. Ovaries were visualized by placing a bright light source behind the fish. No pregnant males were used in stimulus generation or in behavioral assays.

Individuals were randomly assigned to one of two groups: stimulus or focal. Stimulus fish (n = 144; 24 groups of three males and 24 groups of three females) were housed in same-sex tanks (21-l) completely isolated from members of the opposite sex. Focal fish (n = 172) were housed individually (21-l tanks) and isolated from visual and chemical cues of all individuals. Focal fish were allowed 24–72 h to acclimate to their home tank prior to choice tests (see below). No fish were transferred between groups: No stimulus fish were later used as focal fish nor vice versa.

Stimulus Production and Delivery

Stimulus fish were moved from holding tanks to 9.5-l (30 × 15 × 19 cm) stimulus production tanks filled with 9.0 l of artificial seawater (26‰) between 16:00 and 18:00 h. A male and a female were selected at random and placed in separate tanks adjacent to one another. An opaque blind surrounded the two tanks, blocking all visual stimuli except the adjacent tank.

Water was aerated and fish were fed live *Artemia* nauplii (control tanks were aerated and supplied with *Artemia* as well). Tanks were covered with glass to prevent contamination. Gulf pipefish exhibit the majority of their courtship behavior within 4 h of the beginning of the light period (pers. obs. N. Ratterman, A. Jones), so stimulus production and behavioral assays were timed accordingly. Lights were turned off between 23:00 and 24:00. At 08:00 the following day the lights were turned on and stimulus pairs were allowed 1 h to view each other. At 09:00 3 l of water was removed from each stimulus-production tank and placed in an 18-l plastic treatment-specific stimulus container. To control for individual effects, we combined stimulus water from three fish of the same sex for each trial. Hence, a total volume of 9 l of water was collected for male-conditioned water and female-conditioned water. Untreated salt water at the same temperature and salinity was used as a control. Stimulus water was delivered to focal tanks via sterile, thoroughly rinsed (ethanol followed by water) intravenous tubing (Continu-Flo, Baxter Healthcare Corporation, Deerfield, Illinois, USA). The water was sucked into the tubing and gravity was used to generate flow. Roller clamps included with the tubing apparatus regulated flow (1 ml/s). Flow rate was measured by adjusting the flow to fill a 10 ml volume in 10 s. Tubing was secured 3 cm from the end of the tank and 3 cm above the water surface via tube receptacles to keep the delivery tubes stationary (Fig. 1).

For the body size assessment experiment, the same protocol was followed, except that stimulus females

were grouped into large (>102 mm TL) and small (<92 mm TL) categories. These body size cutoffs are one standard deviation greater than or less than the mean of a sample of 140 wild caught females from the same population. A total of three large females, three small females, and six males were used for each day of stimulus generation, but only the female stimulus was used (i.e. these six males were used as visual stimuli for the stimulus-generating females). Thus, in this body size assessment experiment only males served as focal fish and were given a choice between stimulus water from three large and three small females.

Colored dye (Adams Extract, Gonzales, TX, USA) was used to estimate diffusion rate. Two delivery systems were secured at opposite ends of a test tank. The colored dye was delivered to the tank at a rate of 1 ml/s. The dye reached the center of the tank in 240 s. After 900 s the dye had mixed, with the highest concentrations near the point of delivery.

New stimulus water was made every day. Tanks were cleaned with a scrub brush and 95% ethanol and rinsed thoroughly with reverse osmosis water. The tanks were allowed to air dry and were refilled with seawater (26‰). The same cleaning procedure was applied to the delivery system and test tanks.

Choice Tests

Sex recognition

Both sexes were tested for responses to the following cues: (1) male vs. female, (2) male vs. control, or (3) female vs. control. Side of delivery was switched for

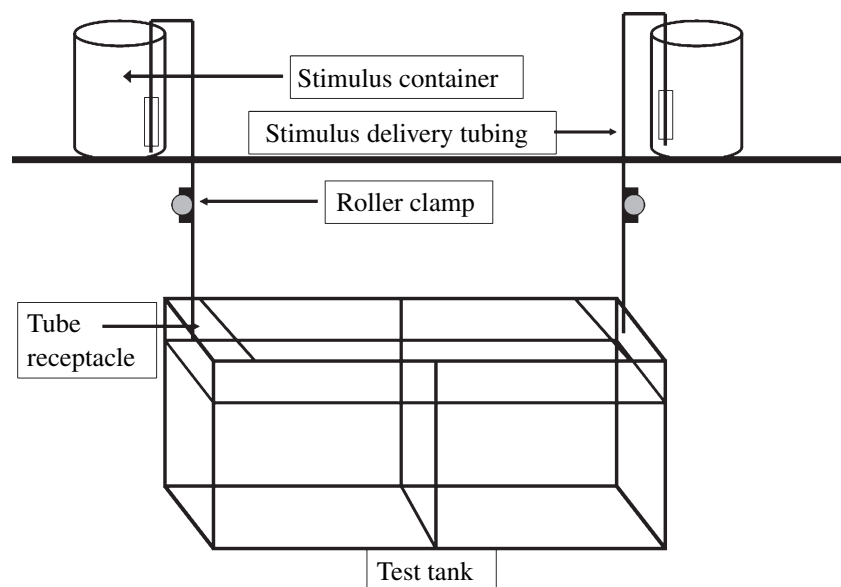


Fig. 1: Experimental set-up for choice tests. Stimuli are produced in separate tanks and then added to containers. Flow is facilitated by gravity and rate is regulated by roller clamps. Tubing is secured at equal distances (3 cm) from the end of the test tank with the tube receptacle, and suspended 3 cm above the water. For the size assessment experiment, the setup is exactly as above but the tank is divided into thirds. See Methods for details.

each trial to control for side biases. A waterproof marker was used to split the area of the tanks in half. Focal fish were given 24–72 h to acclimate to their tank prior to testing (tests were conducted over a series of days and the amount of time spent in the test tank prior to administration of the stimuli did not exceed 72 h). Tanks were moved underneath the stimulus delivery system and tubes were secured in their receptacles (Fig. 1). A visual barrier was secured to the back of the tank to allow for a homogenous background to better track the location of the fish. Each trial started 300 s after positioning the tank under delivery apparatus.

At the initiation of the trial, stimulus water was administered by removing slide clamps from the tubing of both containers simultaneously. The focal fish's initial position was noted and the fish had 600 s to enter the opposite half of the tank. Individuals that failed to cross the centerline in 600 s were excluded from the analysis. Time spent in each half of the tank was recorded as association time, a common metric of mate choice in pipefishes that accurately predicts actual mating preferences (Berglund & Rosenqvist 2001a,b; Berglund et al. 2005). If an individual crossed the centerline only once during the trial and remained motionless for the duration of the trial, it was excluded from the analysis. No individuals were tested twice, nor were any individuals used as both focal tests and stimulus generation.

Body size assessment

For the body size assessment experiment, tanks were divided into three parts: two association zones 10 cm from opposite ends of the tank, as well as one neutral zone (20 cm) between the two association zones. We chose to make the body size assessment tests more stringent than the sex-recognition tests by assigning a neutral area to the middle of the focal tank. Fish were treated exactly as described above, except as follows. A mesh cylinder 20 cm in diameter was used to contain the focal fish in the neutral zone. When the focal fish's tank was moved under the stimulus delivery system, the fish was surrounded by the cylinder and moved to the center of the tank. After 300 s of acclimatization, the two reservoirs of stimulus water were allowed to drip simultaneously into the ends of the tank. Stimulus water was allowed to flow for 240 s before the mesh cylinder was removed. The fish was assumed to have sampled both stimuli at this point and association time in each compartment was recorded for 600 s.

Trials were excluded if individuals did not move from the center zone in 600 s.

Results

Sex Recognition

Males spent significantly more time associating with the female stimulus water than with either the male (Wilcoxon signed-rank test: $Z = 2.11$, $n = 17$, $p = 0.035$) or control water (Wilcoxon signed-rank test: $Z = 2.25$, $n = 17$, $p = 0.025$; Fig. 2). Males showed no preference for male stimulus water over control water (Wilcoxon signed-rank test: $Z = 0.57$, $n = 17$, $p = 0.569$). In contrast, females showed no associative preferences in any of the trials (male vs. control – Wilcoxon signed-rank test: $Z = 0.71$, $n = 12$, $p = 0.480$; male vs. female – Wilcoxon signed-rank test: $Z = 0.70$, $n = 8$, $p = 0.484$; female vs. control – Wilcoxon signed-rank test: $Z = 1.25$, $n = 9$, $p = 0.260$; Fig. 3).

The smaller sample sizes for female responses reflects the fact that most females failed to respond to either stimulus, and thus did not satisfy the predetermined criteria for inclusion of the test (see Methods). Males that were presented with cues from males vs. controls had exclusion rates similar to that of females (Table 1), indicating that males fail to respond to male cues in the same fashion that females fail to respond to all cues in our experimental setup. Approximately two-third of wild caught fish mate in a lab setting, and males responded at approximately this rate when female stimuli were present (Table 1).

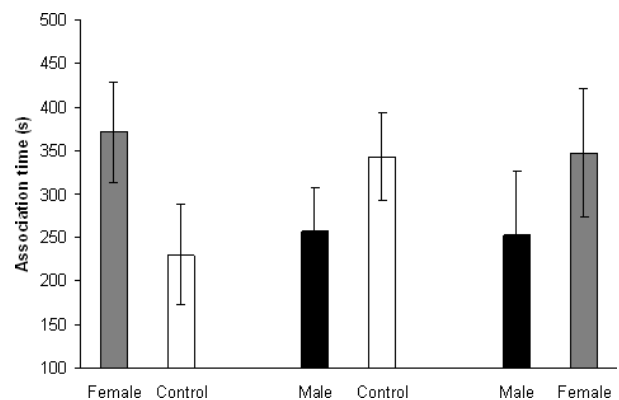


Fig. 2: Sex recognition tests in males revealed significant differences in association time with female vs. male and control, but not male vs. control stimuli. Female treatments are shown in gray bars, males in black bars, and control in white bars. Association time is in seconds.

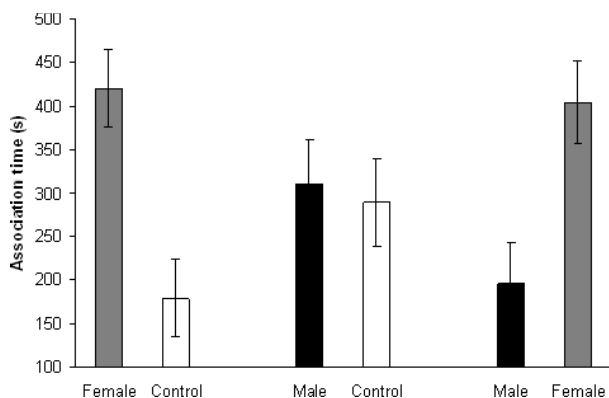


Fig. 3: Sex recognition tests in females revealed no significant preferences. Female treatments are shown in gray bars, males in black bars, and control in white bars. Association time is in seconds.

Table 1: Exclusion rate of trials for the sex recognition experiment. Females did not show preferences for any stimulus, and therefore would not be expected to move in the tank in response to the stimuli. For inclusion in the analysis, individuals were required to become active and cross the center line within 600 s. Tests of male vs. control water with focal males showed approximately the same exclusion rate as all focal female trials

Focal sex	Test	No. excluded	Total trials	% Excluded
Male	Female vs. control	6	24	25%
	Male vs. control	12	27	44%
	Male vs. female	7	24	29%
Female	Female vs. control	7	16	44%
	Male vs. control	9	21	43%
	Male vs. female	15	23	65%

Body Size Assessment

Males showed no preference for large or small female stimuli (Wilcoxon signed-rank test: $Z = 0.205$, $n = 20$, $p = 0.837$).

Discussion

The results of our experiments indicate that male, but not female, *S. scovelli* respond to chemical cues of opposite-sex conspecifics. At first glance these results appear to be consistent with the typical pattern of secondary sexual trait elaboration in females, and male choice based on these traits in sex-role-reversed systems. However, our results also show that males do not distinguish between females of different body size on the basis of chemical cues. Although this result is somewhat surprising given that body size has been shown to be an important

determinant of mate choice in syngnathids based on visual cues (Berglund et al. 1986; Rosenqvist 1990; Silva et al. 2007), it is not unusual. The swordtail *Xiphophorus nigrensis* discriminate based on body size visually, but not chemically (Crapon de Caprona & Ryan 1990). Taken together these two discoveries imply that if the chemical cue has indeed been shaped by sexual selection, then the aspect(s) of female quality that can affect male fitness might not be correlated with body size (e.g. sexual state, overall condition, level of parasite infection, *Mhc* genotype).

The simplest explanation for our results is that males do not release chemical cues that either males or females respond to, and that females gain no particular advantage from responding to chemical cues of other females. While there seems to be no clear rule for which sex emits chemical cues in fishes (Liley 1982; Wyatt 2003; Müller-Schwarze 2006), it has been suggested that a majority of fish chemical signals are emitted by females (Sargent et al. 1998). Wilson (1975) posits that chemical cues are inexpensive to synthesize, so they should be an option for either sex if there is some individual-level benefit to releasing such signals. However, hypothesizing which sex should emit a chemical signal based on relative parental investment (Trivers 1972) may not be an appropriate approach.

Why might it be important for males and not females to identify the sex of a conspecific sender based on chemical cues? Gulf pipefish often live in turbid environments with high densities of sea grass where using vision to locate mates from a distance could be difficult. In such habitats the most effective form of long-distance communication (taken here to mean longer than visual range) may be through chemical signaling (Dusenbury 1992). Theory predicts that either sex can perform mate searching, with the majority of searching being performed by males (Hammerstein & Parker 1987; Kokko & Wong 2007). Additionally, research on *S. typhle* indicates that males may be the searching sex, as females have been observed displaying in groups (Vincent et al. 1994, 1995). If males are the searching sex in the Gulf pipefish as well, then it is a possibility that they are more efficient at finding females if multiple sensory modalities, effective at different distances, are employed (Guilford & Dawkins 1991; Partan & Marler 2005). In this scenario selection could be acting on the male receiver if variation in the ability to detect the female-based cue produces variation in the ability of males to locate a potential mate, which is interesting in the sense that previous work

indicates substantially stronger sexual selection on females than males (Jones et al. 2001).

One way to reconcile the lack of response in females is the possibility that chemical cues from conspecifics may have a priming effect in females vs. a releaser effect in males. In this case, males should respond instantly to the cues, whereas females should need more time and potentially additional sensory stimulation to respond (Wilson & Bossert 1963), possibilities that our experimental design did not accommodate. Similarly, it is possible that the chemical environment of the artificial seawater used did not facilitate effective signaling due to altered pH (Heuschele & Candolin 2007), Ca^{2+} (Hubbard et al. 2002), or environmental contaminants (Fisher et al. 2006). Temperature has recently been shown to impact mate choice in the black-striped pipefish, *S. abaster* (Silva et al. 2007) and might have an influence in the chemical realm, though the temperatures we used were within the natural range at this point in the year (pers. obs. N. Ratterman). Another potential explanation is that females only respond to the cues in specific social settings. Gulf pipefish females aggressively display toward one other and may form dominance hierarchies (pers. obs. N. Ratterman). Perhaps the chemical signals are used intrasexually as dominance badges, as is seen in cockroaches (Moore et al. 1997). Similarly, it is possible that female response to chemical cues is contingent upon stimulation in a non-olfactory sense (e.g. vision), as is common in multimodal communication systems (reviewed in Candolin 2003).

An important implication of our experiments is that chemical signaling may be an important, understudied aspect of reproduction in pipefish. Previous work on sexual selection in pipefishes has focused primarily on visual communication during mate choice (Berglund et al. 1986; Rosenqvist 1990; Berglund & Rosenqvist 1993, 2001a,b). For example, *S. typhle* is a sex-role-reversed species in which females compete strongly for mating opportunities (Berglund & Rosenqvist 1993; Jones et al. 1999). Females exhibit a more conspicuous visual phenotype during courtship than when they are not courting (Berglund et al. 1986; Berglund & Rosenqvist 2001a,b), and males respond behaviorally to this phenotype. In another pipefish, *Nerophis ophidion*, female conspicuousness is even more pronounced. Females are much larger than males, develop a dorsal skin fold during the breeding season, and have blue ornamentation on their head (Berglund et al. 1986; Rosenqvist 1990). Indeed, male *N. ophidion* prefer to mate with larger, more

striking females relative to smaller, less ornamented ones (Berglund et al. 1986; Rosenqvist 1990). While the interpretation of these studies of *S. typhle* and *N. ophidion* focused mainly on visual cues, some of the experiments did use permeable barriers in the mate choice trials and consequently did not address whether the attraction was due to visual cues, chemical cues, or both. Our results indicate that these issues need to be addressed in the future to develop a more robust picture of mate choice in syngnathid fishes.

Sex-role-reversed species have proven to be important models for studying the evolution of sexually-selected traits. Sexual selection involves complex, multivariate suites of traits and preferences (Mead & Arnold 2004) and sexual communication often involves multiple sensory modalities (Partan & Marler 2005). In sex-role-reversed species, the process of mate choice can be sensitive to the local operational sex ratio (Berglund 1993) and the cost of choosing (Berglund 1994). Our results imply that multiple cues in different sensory modalities may be involved in the process of modulating mate choice and suggest that multivariate mate choice in sex-role-reversed species may be a fruitful avenue for future research.

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