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D. Park · J. M. McGuire · A. L. Majchrzak
J. M. Ziobro · H. L. Eisthen

Discrimination of conspecific sex and reproductive condition using chemical cues in axolotls (*Ambystoma mexicanum*)

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Abstract Chemosensory cues play an important role in the daily lives of salamanders, mediating foraging, conspecific recognition, and territorial advertising. We investigated the behavioral effects of conspecific whole-body odorants in axolotls, *Ambystoma mexicanum*, a salamander species that is fully aquatic. We found that males increased general activity when exposed to female odorants, but that activity levels in females were not affected by conspecific odorants. Although males showed no difference in courtship displays across testing conditions, females performed courtship displays only in response to male odorants. We also found that electro-olfactogram responses from the olfactory and vomeronasal epithelia were larger in response to whole-body odorants from the opposite sex than from the same sex. In males, odorants from gravid and recently spawned females evoked different electro-olfactogram responses at some locations in the olfactory and vomeronasal epithelia; in general, however, few consistent differences between the olfactory and vomeronasal epithelia were observed. Finally, post hoc analyses indicate that experience with opposite-sex conspecifics affects some behavioral and electrophysiological responses. Overall, our data indicate that chemical cues from conspecifics affect general activity and courtship behavior in axolotls, and that both the olfactory and vomeronasal systems may be involved in discriminating the sex and reproductive condition of conspecifics.

Keywords Electro-olfactogram · Olfactory · Pheromone · Salamander · Vomeronasal

Abbreviations EOG: electro-olfactogram · VNO: vomeronasal organ

D. Park · J. M. McGuire · A. L. Majchrzak · J. M. Ziobro
H. L. Eisthen (✉)
Department of Zoology, Michigan State University,
East Lansing, MI 48824, USA
E-mail: eisthen@msu.edu
Tel.: +1-517-3531953
Fax: +1-517-4322789

Introduction

Chemosensory signals play important roles in the daily life of many vertebrates. Behavioral and endocrine responses to conspecific chemical cues have been investigated in fishes (Sorensen and Stacey 1999; Stacey et al. 2003), amphibians (Dawley 1984; Rollmann et al. 1999), reptiles (Burghardt 1970; Halpern and Frumin 1979; Mason et al. 1989), and mammals (Meredith 1998; Wysocki and Meredith 1987). In salamanders, conspecific chemical cues are involved in a variety of behaviors, such as individual recognition (Jaeger 1981; Ovaska 1988), discriminating sex and reproductive condition of conspecifics (Marco et al. 1998; Verrell 1985), advertising territories (Chivers et al. 1996; Simons et al. 1994), and modulating general and sexual activities (Houck and Reagan 1990; Park and Park 2002).

The functions of a chemical cue can be studied on two levels. Behavioral studies can demonstrate that an odorant elicits specific behaviors or that exposure to different odorants has different behavioral consequences, and neurobiological studies can examine the physiological underpinnings of these behaviors. Electrophysiological studies of both the olfactory organ and vomeronasal organ (VNO) can provide important insights into the functional separation of the main olfactory and vomeronasal systems, an issue that is still poorly understood, particularly in aquatic vertebrates. To date, few studies integrate across both levels of analysis (Murphy et al. 2001). In addition, more studies of the functional distinction between the olfactory and vomeronasal systems are needed if we are to understand why the vomeronasal system arose in tetrapods (Eisthen 1992, 1997). Although the VNO has been studied for decades, its function relative to the remainder of the olfactory system has not yet been clearly defined for any group of animals (reviewed in Halpern and Martínez-Marcos 2003).

We are investigating the functional significance of chemical signals in axolotls (*Ambystoma mexicanum*),

which are large, non-metamorphosing aquatic salamanders indigenous to two lakes near present-day Mexico City (Brandon 1989). Although axolotls are essentially a subspecies of tiger salamander, *A. tigrinum* (Schaffer 1993), axolotls reproduce more readily in the laboratory than do other ambystomid salamanders. Courtship and mating behaviors are fairly conserved within the family Ambystomatidae, and the extended sequence of nudging and cloacal contact that occurs during mating suggests that chemical signals may play an important role in these behaviors (Armstrong et al. 1989; Arnold 1976; Eisthen and Park 2004; Shoop 1960). In addition, axolotls possess both an olfactory organ and VNO with readily accessible, flat epithelia containing large receptor neurons, making them well suited for electrophysiological studies (Eisthen et al. 1994; Park and Eisthen 2003; Park et al. 2003).

To begin to understand the role of chemical cues in axolotl reproductive behavior, we conducted experiments to determine whether sexually mature male and female axolotls respond differently to odorants from axolotls in different reproductive conditions. Although the natural breeding season for axolotls is not known, data from other ambystomids and from experience rearing axolotls in laboratories suggest that the peak of the breeding season may occur in the spring (Armstrong et al. 1989; Arnold 1976). Females fertilize their eggs internally, then lay most or all of their eggs in a spawn that may contain several hundred eggs. In the laboratory, healthy, well-fed females can be induced to spawn again after 2–4 months (Armstrong et al. 1989), but in the wild females probably spawn less frequently. If so, males may be under considerable pressure to discriminate gravid females from those that have recently spawned or are otherwise not in breeding condition. In contrast, males typically deposit a few spermatophores during each mating bout, and can mate successfully at 2- to 3-week intervals (Armstrong et al. 1989), suggesting that females may not need to discriminate between males that have spawned recently and those that have not.

In this study, we used behavioral observations to determine whether axolotls respond differently to whole-body odorants from conspecifics of differing reproductive status, then used electrical field potential (electro-olfactogram, EOG; Ottoson 1956) recordings to determine whether these behavioral responses are mediated by the olfactory or vomeronasal epithelium. We found that whole-body odorants affect general activity in males and courtship displays in females, and that both the olfactory and vomeronasal systems may play a role in discriminating sex and reproductive status of conspecifics.

Materials and methods

Animals and maintenance

Axolotls were obtained from the Indiana University Axolotl Colony (IUAC) or were derived from parent stock from the IUAC. Animals were kept in aquaria approximately 90 cm long×45 cm wide×30 cm high and were maintained in 100% Holtfreter's solution, the most commonly used medium for maintaining axolotls (Armstrong et al. 1989). Holtfreter's solution contains (mmol l^{-1}): 60 NaCl, 2.4 $NaHCO_3$, 0.67 KCl, 0.81 $MgSO_4$, and 0.68 $CaCl_2$ in dH_2O (pH 7.5); in stock aquaria, but not in experimental situations, 0.0002% NovAqua (Novalek, Hayward, Calif., USA) was added to the Holtfreter's solution to help maintain healthy skin. All aquaria were equipped with a recirculating filter system in which Holtfreter's solution from groups of tanks passed through mechanical and biological filters and an ultraviolet sterilizer before being returned to tanks. To minimize stress, no more than six same-sex individuals were housed in each tank. To prevent exposure to odorants from opposite-sex animals, males and females were kept in separate sets of tanks that did not share a water supply, and separate dip-nets were used for each tank. The temperature of the tanks ranged between 18 and 22°C, and the photoperiod was altered monthly to match that of the animals' native habitat in Mexico City. Axolotls were fed commercial salmon chow (Rangen, Buhl, Idaho, USA) two to five times each week.

Behavioral responses to whole-body odorants from conspecifics

A total of 16 male and 16 female axolotls were initially used as subjects in behavioral tests. One animal run as a male subject was later dissected and found to be female; data from this animal were excluded from all analyses. Stimulus odorants were collected from a total of 8 females and 5 males, and the sex of each of these animals was confirmed either by breeding or by dissection. Although some animals served as both subjects and odorant donors, no animal served as an odorant donor for a trial in which it was a subject. All subjects and odorant donors were removed from stock aquaria and placed in individual bowls at least 1 week prior to use. All tests were conducted in the spring (11 March–4 June 2002 and 17 January–7 March 2003) to coincide with the animals' seasonal reproductive cycle.

For each test, a subject was placed in an individual aquarium (50 cm long×26 cm wide×30 cm high) containing 8 l Holtfreter's solution, and was left to habituate for 12–15 h under a dim red light. After the habituation period, 2 l of stimulus solution were introduced into the aquarium. All activities of each subject were recorded under the same red light for 3.5 h using a low light-level video camera (model WV-BP330; Panasonic USA, Secaucus, N.J., USA) and a time-lapse videocassette recorder (model AG-RT600; Panasonic USA, Secaucus, N.J., USA).

Each animal was exposed to four different stimulus solutions, with the order of exposure determined by a Latin square design. Stimulus solutions consisted of whole-body odorants from same-sex conspecifics and from opposite-sex conspecifics that had spawned recently or not; plain Holtfreter's solution was used as a control. Thus, for example, a male subject was tested with water from other male axolotls, from gravid females, and from recently spawned females, as well as with plain Holtfreter's solution.

Prior to the beginning of the experiment, pairs of male and female odorant donors were placed in tanks together overnight and videotaped so that courtship behavior and spermatophore deposition could be assessed. Females that were used as "recently spawned" odorant donors had laid eggs 1 week to 1 month prior to the beginning of the experiment; females that had not recently spawned were identified as "gravid" if they had the rounded trunk typical of egg-laden axolotls and had not spawned for at least 6 months prior to the beginning of the experiment. "Recently spawned" male odorant donors had laid at least three spermatophores while courting a female during the previous month; males were considered "not recently spawned" if they had not laid spermatophores while courting a female during the previous 6 months.

In all cases, two animals of the same sex and reproductive condition served as odorant donors, and odorants from the two were combined to reduce effects of individual differences. During periods of odorant collection, pairs of stimulus animals were

maintained overnight in individual bowls containing 2 l Holtfreter's solution. Immediately before an experimental trial began, 1 l solution was removed from each bowl and combined before being added to the subject's tank. Stimulus animals were fed between periods of odorant collection so that stimulus solutions would not be contaminated with food or feces. After odorant collection and experimental trials, bowls and aquaria were scrubbed with baking soda and NaCl and thoroughly rinsed with dH_2O.

To quantify the behavioral effects of conspecific odorants, we examined a measure of general activity as well as a measure of courtship activity. To measure general activity, we drew lines on a video monitor to divide each tank into six equal-sized compartments and then recorded the number of times the tip of the subject's snout crossed a line during each 5-min bin. In one trial for one subject, the videotape ended after 175 min; thus, only data occurring during the first 175 min of each trial were included in statistical analyses. We used Student's *t*-test to compare the baseline level of activity in male and female subjects exposed to plain Holtfreter's solution; the raw data were not normally distributed (Shapiro-Wilk test, both $P < 0.01$), but those of log-transformed data were (Shapiro-Wilk test, both $P > 0.72$) and were used for this analysis.

For female subjects, the variances of the activity data were homogeneously distributed (Levene's test, $P = 0.75$) and were analyzed using repeated-measures analyses of variance (ANOVAs). In contrast, the variances of activity data from male subjects were not homogeneous (Levene's test, $P < 0.001$), but the variances of square-root transformed data proved homogeneous (Levene's test, $P = 0.11$); all subsequent analyses of male activity were therefore performed on square-root transformed data.

To measure courtship activity, we examined hula displays, a rhythmic undulation of the posterior parts of the body and tail performed by both males and females; these displays characterize courtship in ambystomids and other salamanders (Arnold 1976). In our tests, we recorded the frequency and duration of hula displays for the five females and five males that performed at least one hula display during any test. Axolotls occasionally perform hula displays in erratic patterns that involve short pauses between displays; in quantifying hula displays, we recorded the beginning of a new bout when an animal ceased to perform the hula display for more than 1 min. For each trial for each animal, the number of hula bouts and the total hula bout duration were used for statistical analyses.

Because the vigor or intensity of hula displays varies considerably among hula bouts, we also quantified the intensity of the display performed during each bout of hula activity. To determine the hula intensity, each bout was assigned a "hula score" according to the maximal level of hula display within that bout. Hula scores were assigned as follows: 1 = only the tip of the tail undulates, and the base of the tail and caudal trunk do not move; 2 = the entire tail, including the base, undulates, but the caudal trunk does not move; 3 = the entire tail and caudal portions of the body undulate within less than 2.5 cm on either side of the main axis of the body; 4 = the entire tail and caudal parts of the body undulate more than 2.5 cm on either side of the main axis of the body. The hula score for each bout was determined by an observer who was blind to the test condition. For each animal, the average hula intensity was calculated by multiplying the average hula score by the average bout duration.

In one trial for one of the subjects that performed hula displays, the videotape ended 15 min before the trial ended; thus, only data occurring during the first 195 min of each trial were included in statistical analyses of hula data. The variances of the data for hula frequency, duration, and intensity were not homogeneously distributed (Levene's test, $P < 0.01$). We therefore analyzed the data using a repeated-measures ANOVA on ranks instead of raw data (Iman 1974 cited in Zar 1996). When this test indicated a significant difference among treatments, Wilcoxon tests were used for post hoc pairwise comparisons of rank-transformed data.

Statistical analyses of behavioral data were conducted using JMP 5.0 (SAS Institute, Cary, N.C., USA) on a Macintosh G4 computer running OSX.

Electrophysiological responses to whole-body odorants from conspecifics

EOG responses were recorded from 11 adult female and 10 adult male axolotls in May 2003. With the exception of 1 female, none of these animals served as subjects in behavioral experiments. The sex of each animal was verified by dissection after recording. All animals were obtained from the Indiana University Axolotl Colony and housed as described above.

Responses elicited by a series of different odorants were recorded. First, in all subjects, an amino acid odorant was used as a positive control. Responses to conspecific odorants were then normalized relative to the magnitude of the response elicited by the amino acid to correct for differences attributable to variation in recording conditions, such as the height of the recording electrode above the sensory epithelium. For the amino acid stimulus, we used 1 mmol l^{-1} L-methionine, because previous studies indicate that methionine elicits fairly large EOG responses in axolotls (Park and Eisthen 2003; Park et al. 2003). In addition, 11 female and 7 male subjects were tested for responses to whole-body odorants from adult male and female conspecifics. To control for effects of body size on odorant stimuli, odorant donors were size-matched: 2 gravid females (mean ± SD = 87.35 ± 24.96 g) and 2 males (mean ± SD = 82.7 ± 9.76 g) served as odorant donors.

Because the preliminary results of our behavioral tests suggested that males responded differently to odorants from gravid and recently spawned females, in 9 of 10 male subjects we also examined EOG responses elicited by odorants from females of differing reproductive condition. For this analysis, two different gravid females (mean body mass ± SD = 119.1 ± 13.58 g) and 2 recently spawned females (mean body mass ± SD = 121.15 ± 10.82 g) were used as odorant donors. Most male subjects ($n = 6$) were tested both with odorants from size-matched females and males and from size-matched gravid and recently spawned females. For female subjects, we did not examine responses to males that had recently laid spermatophores or not, as these stimuli did not produce significantly different results in behavioral tests.

None of the odorant donors served as subjects in electrophysiological experiments. The sex of each odorant donor was confirmed by breeding or dissection. The pH of all stimulus solutions was adjusted to 7.5–7.6 to match the Holtfreter's solution in which axolotls were maintained, and which bathed the nasal cavity during EOG experiments.

Odorant stimuli were collected from axolotls following procedures similar to those described above, with exceptions noted here. Donor animals were housed for 24 h in 1 l Holtfreter's solution, after which approximately 25 ml Holtfreter's solution was withdrawn. Samples were not collected in cases in which the animal had defecated in the bowl. To reduce individual and daily variations in odorant stimuli collected, we made a 1:1 mixture of odorant solutions from two different individuals of the same sex and reproductive condition. Some odorant solutions were used immediately, and others were frozen at −20°C in 30-ml aliquots and used within 3 days of collection. (In preliminary electrophysiological experiments, we found no difference in EOG responses to odorants that had been frozen for 3 days and thawed versus newly collected odorants; in addition, preliminary behavioral experiments indicated that odorants frozen for 2 days were effective stimuli but that odorants frozen for longer periods of time, 8–12 days, were ineffective.) In some experiments, two aliquots of frozen odorants were combined to provide enough stimulus solution to carry out an experiment.

Before surgery, subjects were anesthetized with pH-corrected 0.1% MS 222 (tricaine methanesulfonate, pH 7.5; Sigma Chemical, St. Louis, Mo., USA) in Holtfreter's solution, and immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide, Sigma Chemical, St. Louis, Mo., USA) dissolved in amphibian Ringer's solution (0.1–0.3 mg/100 g body weight, pH 7.6). Supplemental doses of MS 222 were delivered to the gills and additional Flaxedil was injected intramuscularly as necessary throughout the experiment.

The olfactory and vomeronasal epithelia were exposed by removing the tissue dorsal to the nasal capsule. To record electrical field potentials, a glass capillary electrode (100–200 μm tip diameter) was filled with 1% agar in 3 mol l^{-1} KCl bridged to a chloride-coated silver wire. An Ag-AgCl reference electrode was placed under the skin on the head and isolated from both the Holtfreter's and odorant solutions with petroleum jelly. Electrodes were coupled to a differential amplifier (DP-301; Warner Instruments, Hamden, Conn., USA). Signals were digitized via an ITC-18 (Instrutech , Great Neck, N.Y., USA) or Digidata 1322A interface (Axon Instruments, Foster City, Calif., USA), and recorded and analyzed on a Macintosh computer using AxoGraph software (v. 4.4; Axon Instruments, Foster City, Calif., USA). The magnitude of the EOG response was measured as the maximal height of phasic displacement from baseline, and absolute response values in millivolts were obtained by comparison with the deflection elicited by a known voltage.

During each trial, a continuous flow (3.5–4 ml min^{-1}) of Holtfreter's solution bathed the olfactory mucosa. For each EOG recording, 100 μl of room temperature (23–25°C) stimulus solution was injected into the continuous flow of Holtfreter's solution over a 2-s period using a 1-ml syringe. We have previously calibrated the stimulus onset and offset times in this system, and find that odorant arrives at the epithelium approximately 10 s after injection into the carrier stream and remains on the epithelium approximately 2–3 s (Park and Eisthen 2003).

Because the magnitude of EOG responses varies across the sensory epithelium due to such factors as differential access of stimulus solutions and differential distribution of odorant receptors (Mackay-Sim et al. 1982; Mackay-Sim and Shaman 1984), we recorded from several sites on both the olfactory and vomeronasal epithelium. The main chamber of the nasal cavity is almost completely lined with olfactory epithelium except for a strip along the lateral edge, and extends approximately 10 mm anterior-posterior and 2–3 mm medial-lateral in an adult axolotl (Eisthen et al. 1994). To obtain a series of recording locations in the main olfactory organ, we divided the epithelium into anterior, middle, and posterior regions and chose two or three sites within each region for a total of eight recording sites. In contrast, the VNO is quite small even in very large adults, with a sensory epithelium that extends less than 1 mm anterior-posterior and less than 0.5 mm medial-lateral (Eisthen et al. 1994); thus, we were not able to record from many distinct locations within the VNO, and chose one anterior and one posterior site. We were able to reliably locate the same recording sites in different individuals by using such landmarks as the shape and topography of the nasal cavity and the location of prominent blood vessels within the olfactory epithelium. The locations of our recording sites are illustrated in Fig. 1.

The order of sampling the different sites was determined semi-randomly, and varied among subjects. For each subject, we recorded first from the VNO, and the order of sampling the two sites was arbitrary. Next, recordings were obtained from the eight sites on the olfactory epithelium, with the order of sampling randomized across subjects using an eight-sided die. At each site, the EOG response was first optimized by recording responses to L-methionine while adjusting the position of the recording electrode. Next, plain Holtfreter's solution, the carrier solution for all odorant stimuli, was delivered to test for mechanical artifacts and to ensure that all odorant was cleared from the delivery apparatus; if the response elicited by Holtfreter's solution was larger than 10% of the magnitude of the response elicited by L-methionine, adjustments were made until the response elicited by Holtfreter's was minimal. The order of stimulus presentation was determined by throwing a die; note that responses to L-methionine were recorded again along with those to other stimuli, and the initial responses used to optimize recording conditions were not included in the data analysis. To prevent odorant adaptation from occurring, a 4 min inter-stimulus interval was used (Park and Eisthen 2003).

To compare the magnitudes of the EOG responses elicited by different stimuli, the response to each stimulus at each recording site was expressed as a percentage of the EOG magnitude elicited by L-methionine at that site. The means for most such data (36 out of 40 sites) were normally distributed (Shapiro-Wilk test, $P > 0.05$); therefore, parametric statistical tests were used for analysis. Paired *t*-tests were used to compare responses evoked by odorants from members of the same and opposite sex in 7 males and 11 females, and by odorants from gravid and recently spawned females in 9 males.

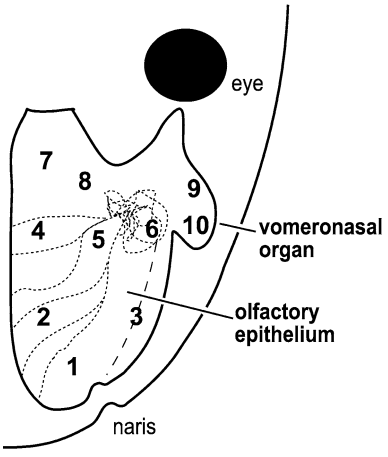

Fig. 1 Electro-olfactogram (EOG) recording sites on the olfactory (*1–8*) and vomeronasal (*9* and *10*) epithelia in axolotls. The vomeronasal organ (VNO) extends laterally from the main chamber of the nasal cavity. Recording site *3* was inside the lateral nasal groove (*dashed line*). *Dotted lines* indicate the position of major blood vessels inside the nasal cavity, which were used to help locate recording sites across subjects

To compare the magnitudes of the EOG responses elicited by L-methionine at different recording sites, we normalized the data for each subject such that the largest response to L-methionine among the ten different recording sites was designated 100%, and the responses at the other nine sites expressed as a percentage of this largest response. The means for most of the resulting data (eight out of ten sites) were normally distributed (Shapiro-Wilk test, $P > 0.05$), so two-tailed *t*-tests were used to compare responses at each recording site to test for sex differences in responses to L-methionine.

We compared the magnitude of the EOG response at each recording site in female subjects that had experience with male conspecifics and those that did not. The means for most of the resulting data (13 out of 20 comparisons) were normally distributed (Shapiro-Wilk test, $P > 0.05$), so parametric statistics (independent-sample *t*-tests) were used in this analysis. Among male subjects, only one had experience with adult females prior to the experiment, so we were not able to analyze the effects of experience on responses in males.

Statistical analyses of electrophysiological data were conducted using SPSS 10.0 (SPSS, Chicago, Ill., USA) on a Dell computer using the Windows XP operating system.

Results

Behavioral responses to whole-body odorants from conspecifics

Figure 2 illustrates general activity during exposure to different odorant stimuli in male and female subjects. General activity during exposure to the control odorant, plain Holtfreter's solution, did not differ between males and females ($t = 0.33$, $df = 27$, $P > 0.75$).

As illustrated in Fig. 2A, the activity of male axolotls did not differ significantly among odorant treatments when subjected to a one-way analysis of variance ($F_{3,56}=2.27$, $P=0.09$). The subjects used in this experiment had varying degrees of sexual experience prior to testing: 1 male was 12 months old at the time of testing, and although sexually mature, had never been paired with a female nor exposed to odorants from adult females; at the other extreme, 1 male had been paired with females 17 times and had spawned twice. Overall, 9 of the 15 subjects had been paired with females prior to testing; of these, 5 had laid spermatophores when paired with females but a successful spawning did not result, and an additional 2 subjects had successfully spawned. We therefore analyzed the data using a series of two-way repeated-measures ANOVA tests to determine whether prior experience being paired with a female, prior experience laying spermatophores while paired with a female, or prior experience spawning contributed significantly to the results obtained.

In the first of these analyses, data from males that had been paired with a female prior to testing ($n=9$) were compared with those from males that had never been exposed to odorants from sexually-mature females ($n=6$). As illustrated in Fig. 2C, comparisons of both odorant treatment and experience are statistically significant (odorant treatment: $F_{3,52}=2.82$, $P=0.048$; experience: $F_{1,52}=5.18$, $P=0.027$); the interaction between these variables is not significant ($F_{3,52}=0.70$, $P=0.55$). Post hoc Tukey tests were used to test for pairwise differences among treatments within a group, as well as between experienced and inexperienced males for each treatment. None of these tests revealed statistically significant differences (all $P>0.05$).

An additional analysis was conducted to compare activity of males that had laid spermatophores while paired with a female ($n=7$) with those that had not ($n=8$), over all odorant treatments. In this analysis, neither treatment nor experience nor the interaction of the two contributed statistically significant results (odorant treatment: $F_{3,52}=2.14$, $P=0.11$; experience: $F_{1,52}=2.14$, $P=0.15$; interaction: $F_{3,52}=0.11$, $P=0.96$). Because only two male subjects had successfully spawned prior to testing, a two-way ANOVA treating spawning success and odorant treatment as factors was not possible. We therefore eliminated data from the two subjects that had spawned and conducted a one-way ANOVA on the data from the remaining subjects ($n=13$) to determine whether prior experience spawning alters behavioral responses to conspecific odorants. In this analysis, as in the analysis of the complete data set, differences among odorant treatments in males were not significant ($F_{3,48}=2.22$, $P=0.10$).

As illustrated in Fig. 2B, the activity of female axolotls did not differ significantly among odorant treatments when subjected to a one-way analysis of variance ($F_{3,60}=0.38$, $P=0.77$). As with males, the female subjects used in this experiment had varying degrees of

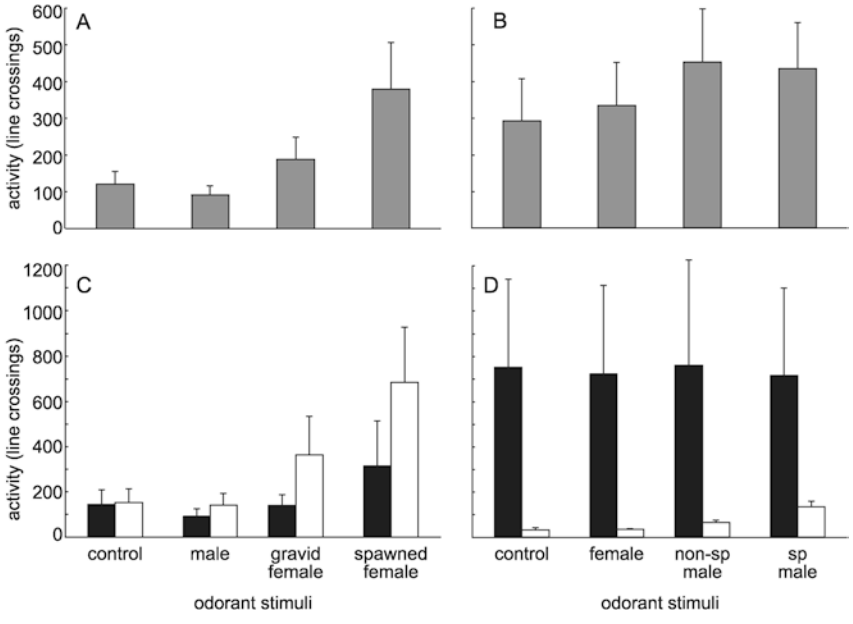

Fig. 2 General activity of male (**A**, **C**) and female (**B**, **D**) axolotls in response to whole-body odorants, measured as the number of line crossings during 175 min of odorant exposure. All subjects were tested with plain Holtfreter's solution (*control*), and males were tested with odorants from other males (*male*) and from females that had spawned recently (*spawned female*) or not (*gravid female*). Female subjects were tested with odorants from other females (*female*) and from males that had laid spermatophores recently (*sp male*) or not (*non-sp male*). In both males (**A**, $n=15$) and females (**B**, $n=16$), activity level did not differ across treatment conditions ($P=0.09$ and $P=0.77$, respectively). **C** Activity levels differed between males that had prior experience with adult female axolotls (*black bars*, $n=9$) and those had no experience with females (*white bars*, $n=6$); activity level was also found to differ significantly among treatment conditions (both $P<0.05$). **D** Activity level differed significantly ($P=0.02$) between females that had spawned at least once prior to testing (*black bars*, $n=5$) and those that had never spawned (*white bars*, $n=11$), but did not differ among treatment conditions ($P=0.91$)

sexual experience prior to testing: 5 of 16 subjects had spawned prior to testing; 3 other females had been paired with males that laid spermatophores, but did not subsequently spawn; 6 additional females had been paired with males, but no signs of courtship or sexual activity were observed; and 2 females had not been paired with a male nor exposed to odorants from adult male conspecifics prior to testing. We further analyzed female activity data to determine whether any of these types of experience contributed significantly to the results obtained.

Only two subjects had no experience with adult males prior to testing; we therefore eliminated data from these two subjects from analysis and conducted a one-way ANOVA on the data from the remaining subjects ($n=14$). This analysis did not reveal a significant effect of odorant treatment among females that had prior experience with males ($F_{3,52}=2.22$, $P=0.10$). An additional analysis was conducted to compare activity of females with which a male had laid spermatophores prior to testing ($n=8$), regardless of whether or not a successful spawning resulted, compared with females with which a male had not laid spermatophores, including females that had no prior experience with males ($n=8$), over all odorant treatments. In this analysis, neither treatment nor experience nor the interaction of the two contributed statistically significant results (odorant treatment: $F_{3,56}=0.36$, $P=0.78$; experience: $F_{1,56}=0.56$, $P=0.43$; interaction: $F_{3,56}=0.26$, $P=0.87$).

Finally, data from females that had spawned prior to testing ($n=5$) were compared with those of females that had never spawned ($n=11$). As illustrated in Fig. 2D, activity levels of females that had spawned prior to testing were much higher than those of females that had not spawned. Although the effect of experience was statistically significant ($F_{1,56}=6.25$, $P=0.02$), neither odorant treatment nor the interaction proved significant (treatment: $F_{3,56}=0.36$, $P=0.91$; interaction: $F_{3,56}=0.19$, $P=0.90$).

A minority of animals performed hula displays during tests: five males (33%) and five females (31%) performed hula displays during experimental trials. Of the six male subjects that had no previous experience with females, three performed hulas; of the five that had laid spermatophores but not spawned, two performed hulas. Neither of the males that had spawned prior to testing performed hula displays in any treatment condition. We found no evidence of a relationship between experience with females and the incidence of hula displays in male subjects (Fisher's exact test, two-tailed, $P=0.33$). Of the five female subjects that had spawned prior to testing, two performed hula displays; of nine other females that had been paired with males, two performed hula displays; and one of the two females that had not previously been paired with a male performed hula displays in our tests. As with male subjects, we found no evidence of a relationship between experience with males and the incidence of hula displays in females ($\chi^2=0.2$, $df=2$, $P=0.90$).

Among the five males that performed hula displays in tests, only one performed hula displays in more than one testing condition. Two animals performed hula displays in response to stimulation by odorants from gravid females; two performed hula displays in response to odorants from recently spawned females; one performed hula displays in response to odorants from other males; and two performed hula displays in response to control odorants. The probability of a hula display occurring did not differ significantly across treatment conditions ($\chi^2=0.70$, $df=3$, $P=0.87$). As illustrated in Fig. 3, the frequency (Fig. 3A; $F_{3,16}=0.34$, $P=0.80$), duration (Fig. 3C; $F_{3,16}=0.23$, $P=0.87$) and intensity (Fig. 3E; $F_{3,16}=0.17$, $P=0.92$) of hula displays by males did not differ across treatment conditions.

The five females performing hula displays in tests did so only in response to stimulation with odorants from conspecific males: one performed hula displays in response to odorants from males that had not laid spermatophores within the previous 6 months; three performed hula displays in response to odorants from males that had recently laid spermatophores; and one performed hula displays in both male odorant treatments. Among females, the probability of a hula display occurring differed significantly across treatment conditions ($\chi^2=12.7$, $df=3$, $P=0.005$).

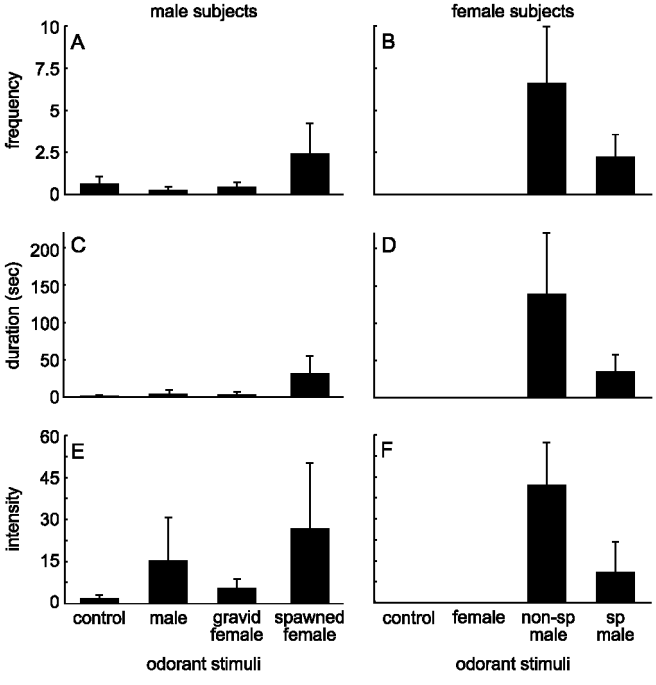

Fig. 3 Courtship behaviors by male (*left panels*, $n=5$) and female (*right panels*, $n=5$) axolotls while exposed to different odorant treatments. Graphs illustrate the frequency (**A**, **B**), duration (**C**, **D**), and intensity (**E**, **F**) of the hula display, a rhythmic undulation of the caudal trunk and tail. Exposure to whole-body odorants from male conspecifics increases frequency (**B**), duration (**D**), and intensity (**F**) of hula displays in females (one-way ANOVA, in all cases $P<0.01$), but hula displays in males (**A**, **C**, **E**) are not affected by conspecific odorants (one-way ANOVA, in all cases $P>0.8$)

As illustrated in Fig. 3, the frequency (Fig. 3B; $F_{3,16} = 5.39$, $P = 0.009$), duration (Fig. 3D; $F_{3,16} = 5.39$, $P = 0.009$) and intensity (Fig. 3F; $F_{3,16} = 5.83$, $P = 0.007$) of hula displays differed significantly across stimulus conditions. Post hoc tests indicated no differences in frequency, duration, or intensity of hula displays in response to stimulation with odorants from males that had recently laid spermatophores and those that had not (in all cases $P > 0.2$).

Electrophysiological responses to whole-body odorants from conspecifics

Examples of EOG responses elicited by each odorant are illustrated in Fig. 4. Responses elicited by L-methionine and whole-body odorants from males and females are shown in representative recordings from male (Fig. 4A, B) and female axolotls (Fig. 4C, D), at site 6 in the olfactory epithelium (Fig. 4A, C) and at site 10 in the vomeronasal epithelium (Fig. 4B, D).

L-Methionine evoked responses from both the olfactory and vomeronasal epithelia. As depicted in Fig. 5, the magnitude of the response varied across the olfactory epithelium, but did not differ between males (Fig. 5A) and females (Fig. 5B) at most recording sites. In the olfactory epithelium, the largest responses were recorded at sites 2 and 4, and the smallest at sites 1, 3, and 6. In the VNO, responses to 1 mmol l^{-1} L-methionine were smaller than those recorded in the olfactory epithelium (compare also Fig. 4A with B, and Fig. 4C with D). EOG responses elicited by 1 mmol l^{-1} L-methionine were significantly larger in the VNO of females than of males (both $P < 0.04$).

Odorants from conspecifics evoked EOG responses in the olfactory and vomeronasal epithelia in both male and female subjects, as illustrated in Figs. 6 and 7. In male subjects, the largest EOG responses were evoked by odorants from females (see also Fig. 4A, B), while odorants from males evoked the largest responses in female subjects (see also Fig. 4C, D). In male subjects, responses evoked by female odorants were significantly

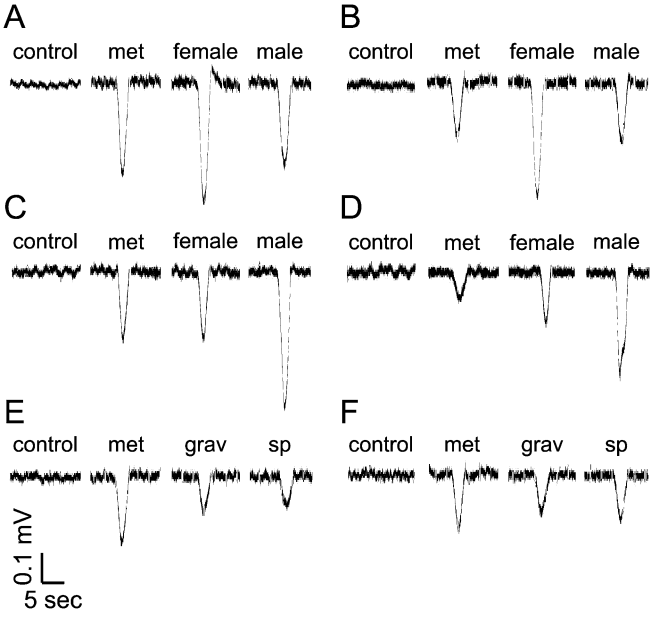

Fig. 4 Representative EOG responses recorded from the olfactory (**A**, **C**, **E**) and vomeronasal (**B**, **D**, **F**) epithelia in male (**A**, **B**, **E**, **F**) and female (**C**, **D**) subjects. The labels above the responses indicate the odorant stimulus used to elicit the response. **A** Responses recorded from a male axolotl at site 6 in the olfactory epithelium in response to the carrier solution (*control*) and to 100 µl of 1 mmol l^{-1} L-methionine (*met*), whole-body odorants from female axolotls (*female*), and whole-body odorants from size-matched males (*male*). **B** Responses to the same stimuli recorded from site 10 in the vomeronasal epithelium of the same subject. **C**, **D** Responses to the same odorants recorded from a female axolotl at site 6 in the olfactory epithelium and site 10 in the vomeronasal epithelium, respectively. In both males and females, the EOG responses were evoked by odorants from the opposite sex. **E**, **F** EOG responses evoked by the carrier solution and methionine as well as whole-body odorants from gravid (*grav*) and recently spawned (*sp*) females recorded at site 5 in the olfactory epithelium (**E**) and site 9 in the vomeronasal epithelium (**F**) from a male axolotl. Odorants from gravid females elicited larger EOG responses at site 5 than did odorants from recently spawned females; the reverse was observed at site 9

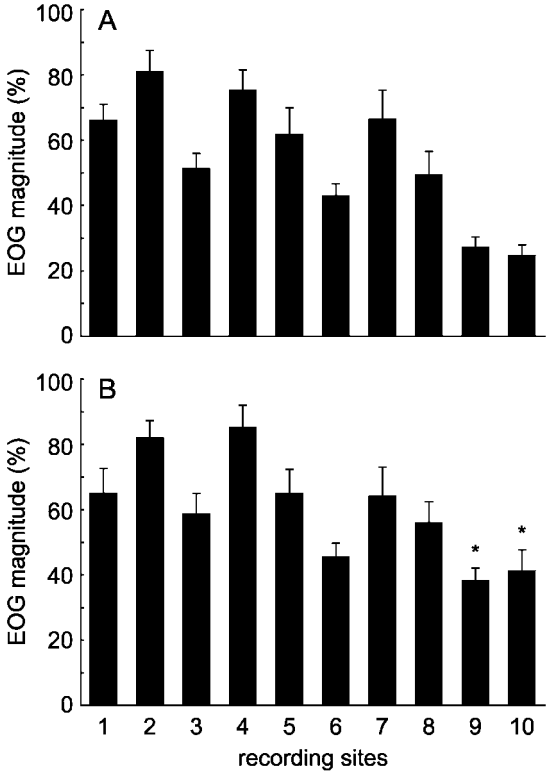

Fig. 5 EOG responses elicited by L-methionine in male (**A**, $n = 10$) and female (**B**, $n = 11$) and axolotls. Data were normalized within individuals such that the largest EOG response at any recording site was designated 100%, and the magnitudes of the remaining EOG responses are expressed as a percentage of this magnitude. In the olfactory epithelium (sites *1–8*), the magnitude of the response evoked by methionine varies, but is largely similar between males and females. In the VNO (sites *9* and *10*), however, EOG responses are larger in females than in males (*asterisk*: $P < 0.05$)

larger than those evoked by male odorants at four of eight sites in the olfactory epithelium (sites 1, 2, 6, and 7) and at both sites in the VNO (Fig. 6A; paired *t*-test, for all cases $P < 0.02$). Among females, responses evoked by male odorants were significantly larger than those evoked by female odorants at all but site 3 in the olfactory epithelium (Fig. 6B; paired *t*-test, for all cases $P < 0.05$) and at both sites in the VNO (Fig. 6B; both $P < 0.05$).

Of the 10 male subjects in these experiments, 1 had spawned successfully and the other 9 had no experience with female conspecifics prior to testing; thus, we did not have sufficient data to be able to analyze the effect of sexual experience on EOG responses in males. However, of the 11 female subjects, 4 had been paired with males prior to testing, resulting in one successful spawning (by the female that also served as a subject in the behavioral experiment). We compared EOG responses at all 10 recording sites in females that had previously been paired with conspecific males ($n = 4$) with those in females that had never been paired with a male ($n = 7$). In both experienced and naïve females, responses to male body odorants were larger than responses to female body odorants, and responses to both of these odorants were larger than responses to methionine. Interestingly, the response magnitudes at some sites differed between experienced and naïve females.

As illustrated in Fig. 7A, responses to methionine did not differ at any site in experienced and naïve females (paired *t*-test, for all cases $P > 0.25$). Responses to whole-body odorants from both female (Fig. 7B) and male (Fig. 7C) conspecifics differed at sites 1 and 2 in the

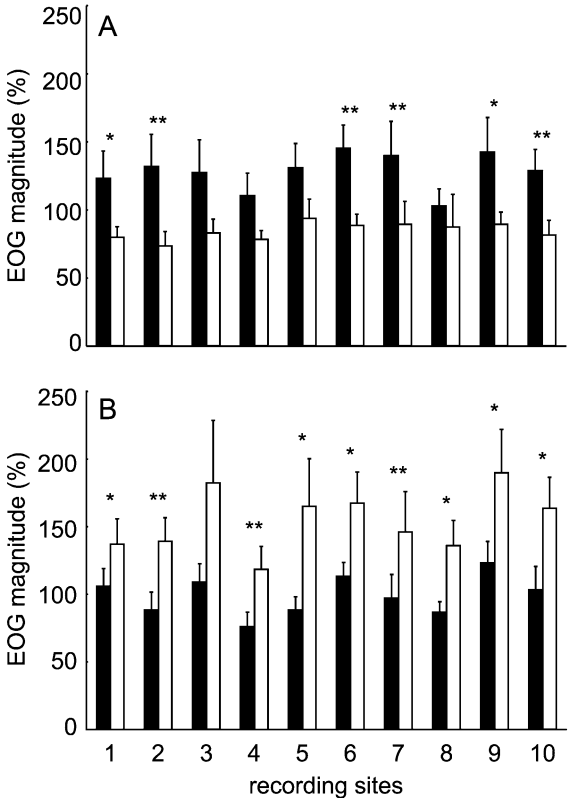

Fig. 6 EOG responses to odorants from size-matched conspecifics at various recording sites in the main olfactory (*1–8*) and vomeronasal epithelium (*9–10*) of male (**A**, $n = 7$) and female (**B**, $n = 11$) axolotls. In this and all subsequent figures, the magnitude of the EOG response at each site is expressed as a percentage of the EOG magnitude elicited by 100 µl of 1 mmol l^{-1} L-methionine at that site. Odorants from females (*black bars*) and males (*white bars*) induce larger EOG responses from the opposite sex in both the olfactory organ and VNO. *Asterisk*: significant difference ($P < 0.05$) in a paired *t*-test; *double asterisks*: $P < 0.01$ in a paired *t*-test

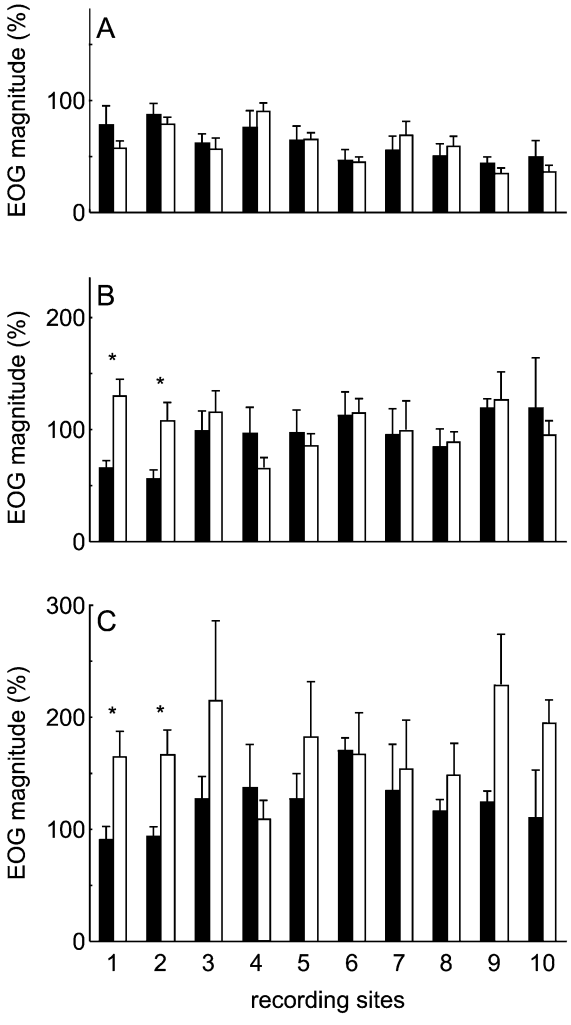

Fig. 7 EOG responses at recording sites on the main olfactory (*1–8*) and vomeronasal epithelium (*9–10*), recorded from female axolotls that had prior experience with conspecific males (*black bars*, $n = 4$) or were naïve with respect to males (*white bars*, $n = 7$). **A** The magnitude of the EOG response elicited by methionine did not differ between experienced and naïve females (all $P > 0.25$). **B** The magnitude of the EOG response to whole-body odorants from female conspecifics differed at sites *1* and *2* in the olfactory epithelium (*asterisk*: *t*-test, both $P < 0.05$), with naïve females producing larger EOG responses to female odorants at these two sites. **C** The magnitude of the EOG response to whole-body odorants from males differed at sites *1* and *2* in the olfactory epithelium (*asterisk*: *t*-test, both $P < 0.05$), with naïve females producing larger EOG responses to male odorants at these two sites

olfactory epithelium (*t*-test, for all cases $P < 0.05$). At both of these sites, EOG responses elicited by conspecific odorants were larger in naïve than experienced females, but differences in EOG responses elicited by conspecific odorants did not differ between naïve and experienced females at other sites in the olfactory epithelium (*t*-test, for all cases $P > 0.15$). In the VNO, responses to male body odorants appeared to be larger in naïve than in experienced females, but this difference was not statistically significant (*t*-test, $P = 0.06$ at site 9 and $P = 0.07$ at site 10); differences in the magnitude of the responses elicited by female body odorants did not differ between naïve and experienced females (paired *t*-test, both $P > 0.5$).

Whole-body odorants from both gravid and recently spawned females induced responses from both the olfactory and vomeronasal epithelia in males, as shown in Fig. 4E and F and in Fig. 8. At most recording sites, the magnitude of the responses elicited by these odorants did not differ; however, differential responses were recorded at two sites. Specifically, at site 5 in the olfactory epithelium, the response elicited by odorants from gravid females was significantly larger than that elicited by odorants from recently spawned females ($t = 2.63$, $df = 8$, $P = 0.03$). In the VNO, the opposite trend was observed: odorants from gravid females elicited smaller responses than did those from recently spawned females, and this difference in response magnitude at site 9 was significant ($t = 3.55$, $df = 8$, $P < 0.01$). These differences are also illustrated in the samples of recordings from sites 5 and 9 shown in Figs. 4E and 5F, respectively.

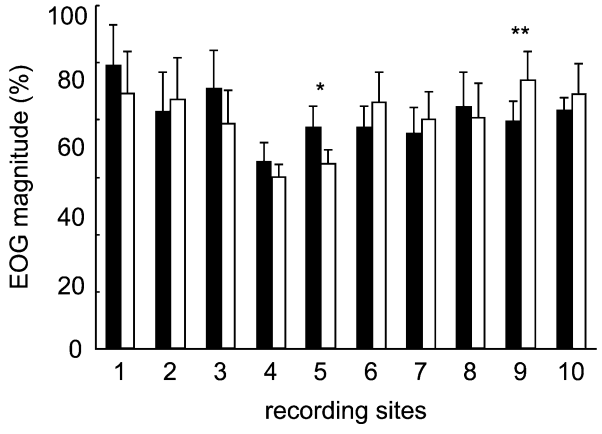

Fig. 8 EOG responses to odorants from gravid and recently spawned females at various recording sites on the main olfactory (*1–8*) and vomeronasal epithelium (*9–10*) of male axolotls ($n = 9$). The magnitude of the EOG response elicited by odorants from gravid females (*black bars*) at site *5* in the olfactory epithelium is greater than that elicited by odorants from recently spawned females (*white bars*). *Asterisk*: significant difference (paired *t*-test, $P < 0.05$). In contrast, the magnitude of the EOG response elicited by odorants from recently spawned females is greater than that elicited by odorants from gravid females at site *9* in the vomeronasal epithelium. *Double asterisk*: significant difference (paired *t*-test, $P < 0.01$). At other recording sites, EOG responses elicited by odorants from gravid and recently spawned females are similar

Discussion

The results of the present study indicate that adult axolotls can distinguish the sex of conspecifics based solely on chemosensory cues. Some differences in physiological responses to odorants from gravid and recently spawned females were observed in males, suggesting that male axolotls may also be able to determine the reproductive status of conspecific females based on odorant cues. In addition, our data indicate that experience with opposite-sex animals alters behavioral and physiological responses to odorants from conspecifics.

In the behavioral experiment reported here, we presented subjects with four different odorant treatments and recorded both the level of general activity and of a courtship behavior, the hula display. The analyses of these two measures revealed different effects of odorant exposure on male and female axolotls, and will be discussed separately.

In male axolotls, exposure to whole-body odorants from conspecific females led to an increase in general activity, relative to the level recorded when males were exposed to odorants from other males or to the Holtfreter's solution control (Fig. 2). The level of activity appeared to be somewhat greater when males were exposed to odorants from recently spawned females versus gravid females, although this difference was not statistically significant with our relatively small sample size. An increase in activity has been described in male goldfish (*Carassius auratus*) exposed to odorants from non-reproductive females, and has been interpreted as indicating that males seek to avoid non-reproductive females in favor of searching for reproductive females (Bjerselius et al. 1995).

We found that males that had no prior experience with adult females were more active than experienced males when exposed to odorants from females (Fig. 2C), regardless of whether the latter animals had engaged in courtship or had spawned. We do not know whether the effect we observed requires behavioral interactions with females, or simply exposure to chemical cues from females; because of our housing regime, males that had not been paired with an adult female prior to testing would have no experience with odorants from adult females.

Among female subjects, exposure to odorants from males did not appear to affect general activity, and we did not observe any difference in activity in response to odorants from males that had or had not recently laid spermatophores (Fig. 2B, D). We found that females that had previously spawned displayed much higher levels of activity in all testing conditions than did females that had not spawned, regardless of other experience with males among the latter group. We propose several explanations, which are not mutually exclusive. Perhaps females that are more active are more likely to spawn; conversely, perhaps spawning changes the physiology of females such that they become more

active. It also seems possible that the healthiest subjects had spawned prior to testing, and that healthier animals may be more active. Nevertheless, all subjects ate well and none had visible signs of illness, suggesting that health did not vary dramatically among subjects.

Overall, measurements of activity levels suggest that males can discriminate the sex of odorant donors, but do not indicate whether either males or females can distinguish the reproductive status of opposite-sex conspecifics based on chemical cues. In other amphibians, chemical cues that serve as sex attractants have also been shown to elicit locomotor activity; for example, when male red-spotted newts (*Notophthalmus viridescens*) are exposed to odorants from female conspecifics, most males begin to move within minutes and eventually approach the odorant source (Park and Propper 2001). Similarly, female magnificent tree frogs, *Litoria splendida,* will approach a gauze pad to which a male pheromone has been applied (Wabnitz et al. 1999).

Approximately one-third of our subjects performed hula displays in at least one testing condition. We found no evidence that prior experience with the opposite sex influences the probability of performing hula displays in either males or females. Male subjects performed hula displays in all odorant treatment conditions, and no differences in frequency, duration, or intensity of hula displays were observed. In contrast, female subjects performed hula displays only when exposed to odorants from males (Fig. 3). Although the frequency, duration, and intensity of hula displays in response to odorants from males that had recently laid spermatophores appear to be lower than in response to odorants from males that had not, this difference was not statistically significant.

The function of the hula display in female axolotls is not clear, but in both axolotls and tiger salamanders, hula displays are observed after females pick up spermatophores (Arnold 1976; Eisthen and Park 2004). Overall, the observation that both experienced and inexperienced females perform hula displays when exposed to male odorants suggests that odorants from male axolotls could function to increase sexual receptivity in females. Similar effects have been described in other salamanders that have been studied. For example, in two species of plethodontid salamanders, *Desmognathus ochrophaeus* and *Plethodon jordani*, male pheromones increase female receptivity, making females more likely to mate with the courting male (Houck and Reagan 1990; Rollmann et al. 1999). In red-spotted newts, unreceptive females often become receptive when exposed to male pheromones (Rogoff 1927).

Given that female axolotls can probably only spawn every few months, we expect that males are under pressure to discriminate between gravid females and those that have spawned recently. Indeed, behavioral studies demonstrate that Western redback salamanders, *P. vehiculum*, and Dunn's salamanders, *P. dunni*, can discriminate between gravid and non-gravid females of equal body size based solely on chemical cues (Marco et al. 1998). In contrast, males seem to be able to deposit spermatophores as often as every few days, and we expect that females would not discriminate between males that have deposited spermatophores recently and those that have not. Nevertheless, we did not observe clear behavioral differences demonstrating that either male or female axolotls respond differently to chemical cues from conspecifics that have spawned recently or have not.

In electrophysiological experiments, we were able to record robust responses throughout the olfactory and vomeronasal epithelia to all odorants tested, and responses were largest in response to stimulation with whole-body odorants from the opposite sex. These results suggest that chemical cues from conspecifics are salient odorants for axolotls. A sexually dimorphic response to whole-body odorants from conspecifics has also been reported in the main olfactory bulb of female Northern crested newts (*Triturus cristatus*; Cedrini and Fasolo 1971).

We found that all sites in the olfactory and vomeronasal epithelia of both males and females respond to an amino acid, L-methionine. The magnitude of the EOG response elicited by L-methionine differs across the olfactory epithelium: in general, the largest EOG responses were recorded at sites in the medial nasal cavity (sites 2, 4, and 7), and the smallest at lateral sites (3 and 6). Differences in EOG response magnitude may be due to differences in the placement of the recording electrode relative to the sensory epithelium, differences in the density of receptor neurons at different locations, or differences in the distribution of odorant receptors. Because we delivered solutions through the naris we would expect that if differences in stimulus access explained much of the variation in response magnitudes that we observed, we would find that the largest responses were recorded at anterior locations, but this is not the case. Nevertheless, we cannot dismiss the possibility that differences in stimulus access or electrode placement contributed to the variability observed. On the other hand, differences in EOG magnitude attributable to differences in density of olfactory receptor neurons or distribution of olfactory receptors have been documented in tiger salamanders (Kauer 2002; Mackay-Sim et al. 1982; Mackay-Sim and Patel 1984), and the relatively large EOG responses elicited by L-methionine at medial sites may be due at least in part to a higher density of olfactory receptor neurons that respond to this stimulus.

Curiously, we found that responses elicited by L-methionine in the VNO are larger in females than in males. Although it is tempting to interpret this result as suggesting that the distribution of receptors for L-methionine differs in the vomeronasal epithelium of males and females, other explanations cannot be ruled out at present. First, the shape of the head and nasal cavities differs between males and females, which could lead to differences in electrode access or flow of stimulus solution. In addition, we recorded large individual differences in overall EOG response magnitude, and

normalized responses to L-methionine relative to the largest recorded response to L-methionine within each individual. Thus, we cannot be certain whether the VNO of females is more sensitive to L-methionine than is that of males (as is suggested in Fig. 5) or whether females are less sensitive to L-methionine across all recording sites, so the difference in responding between the olfactory and vomeronasal epithelia is less pronounced in females (as is suggested in Fig. 4).

Peripheral olfactory responses have been shown to differ with experience in salmon (*Oncorhynchus kisutch*; Nevitt et al. 1994), rabbits (*Oryctolagus cuniculus*; Semke et al. 1995), and mice (*Mus musculus domesticus*; Wang et al. 1993), and a similar phenomenon may occur in axolotls. Specifically, we found that EOG responses to conspecific odorants at anterior locations are larger in females that have no experience with adult males than in those that do. This difference cannot be attributed to our practice of normalizing responses relative to the response elicited by L-methionine, as the magnitude of these responses did not differ between experienced and inexperienced females (Fig. 7A). This difference is probably also not due to differences in the maturity or reproductive state of the two groups of females, as all but one were hatched within 1 month of each other.

Previous studies have demonstrated that sensitivity in olfactory receptor neurons is enhanced as a result of experience, whereas we observed that EOG response magnitude decreases as a result of experience. In addition, previous studies demonstrate that sensitivity to an odorant is enhanced as a result of experience with that particular odorant; in contrast, we found that responses to both male and female whole-body odorants differ in females as a result of experience with adult males, even though all our subjects have considerable experience with odorants from adult females. We cannot at present discriminate among three possible explanations for these discrepancies between our results and those of previous studies. First, we examined responses to complex mixtures, whole-body odorants from conspecifics, whereas previous studies have examined responses to single odorants that do not have an inherent behavioral significance for the species studied. Thus, the complexity and biological relevance of the odorants examined differed between previous studies and our study. Second, the differences we describe appear to be the result of experience with the opposite sex, whereas previous studies have examined changes in sensitivity as a result of different types of experience, including home-stream imprinting (Nevitt et al. 1994), prenatal exposure (Semke et al. 1995), and exposure as adults (Wang et al. 1993). Thus, the behavioral context for learning differed between previous studies and our study. Finally, ours is the first study to document a change in peripheral olfactory responding as a result of experience in an amphibian, and the nature of such changes may differ across taxa.

We found that EOG responses to male odorants are larger in the vomeronasal epithelium of inexperienced than experienced females. Although this difference is not statistically significant at the 0.05 level, our sample sizes were small and the contrast between the two groups is striking (see Fig. 7C). This result is intriguing, for it suggests that experience with conspecific males alters vomeronasal responses only to male odorants, whereas in the anterior olfactory epithelium we found that responses to both male and female odorants were altered in experienced females. Additional studies are clearly needed to investigate this phenomenon further. Behavioral studies with mammals have suggested that experience can alter olfactory, but not vomeronasal, responses to conspecific odorants (reviewed in Halpern and Martínez-Marcos 2003), but experience-related changes in responding in the vomeronasal epithelium have not yet been described in any species.

In males, odorants from gravid and recently spawned females elicited differential responses from one location each in the olfactory and vomeronasal epithelia. What is the significance of the fact that a minority of sites responded differentially to these two odorants? The results of our behavioral experiment suggest that males may respond differently to odorants from gravid and recently spawned females, but statistically significant differences were not observed. Perhaps the differences we observed in our EOG recordings were essentially random, or do not translate into differences in behavior; indeed, if corrections for multiple comparisons are performed, almost none of the results of our *t*-tests would be considered significant. On the other hand, our stimuli consisted of complex mixtures of odorants, and one might expect that a subset of these components would elicit robust responses from almost any location in the nasal sensory epithelium. Thus, it seems possible that differences in responding to cues signaling female reproductive status were overshadowed by responses to other components of the odorant mixture at most locations. Given that responses to odorants differ across the olfactory epithelium, it also seems possible that the chemicals signaling the reproductive status of females elicit larger responses in a few regions of each epithelium.

The chemical cues that axolotls use to discriminate sex of conspecifics may be derived from one or more possible sources. First, axolotls and other ambystomids have prominent cloacal glands. Cloacal glands are an established source of sex pheromones in other salamanders, including Northern crested newts, *T. cristatus* (Malacarne et al. 1984), red-bellied newts, *Cynops pyrrhogaster* (Kikuyama et al. 1995), sword-tail newts, *C. ensicauda* (Kikuyama et al. 1997; Yamamoto et al. 2000), and red-spotted newts, *N. viridescens* (Park and Propper 2002). Second, steroid hormone metabolites may function as chemical cues in axolotls. Behavioral and endocrine responses to steroid hormone metabolites that act as odorants have been reported in several teleosts (reviewed in Stacey et al. 2003), including goldfish, *C. auratus* (Sorensen and Stacey 1999), round gobies, *Neogobius melanostomus* (Murphy et al. 2001), and the cyprinid fish *Barilius bendelisis* (Bhatt and Sajwan 2001), as well as in Italian crested newts, *Triturus carnifex*

(Cobbetti and Zerani 1992; Malacarne and Giacoma 1986). Finally, species-typical bile acids have been shown to serve as potent odorants in lampreys and in teleost fishes (Døving et al. 1980; Li et al. 1995; Michel and Lubomudrov 1995; Zhang et al. 2001), and a bile acid has recently been shown to serve as a sex pheromone in lampreys, *Petromyzon marinus* (Li et al. 2002). Like all vertebrates, salamanders produce bile acids (reviewed in Hoshita 1985; Une and Hoshita 1994), although the possibility that bile acids function as odorants in aquatic salamanders has not yet been investigated.

We did not observe dramatic differences in responding between the olfactory and vomeronasal epithelia. Based primarily on studies with rodents, many researchers have suggested that the VNO is specialized for detection of pheromones, a hypothesis that implies that the olfactory epithelium should not respond to pheromones and that the VNO should be minimally responsive to general odorants. Nevertheless, vomeronasal neurons in mice (*M. m. domesticus*) respond to general odorants as well as pheromones (Sam et al. 2001; Trinh and Storm 2003), and some pheromone responses in mammals are mediated by the olfactory, not vomeronasal, system (Cohen-Tannoudji et al. 1989; Dorries et al. 1997; Sipos et al. 1995; Swann et al. 2001). Furthermore, behavioral, electrophysiological, and biochemical studies have demonstrated unambiguously that the vomeronasal system of garter snakes (*Thamnophis sirtalis*) responds to prey odorants (reviewed in Halpern and Kubie 1984; Halpern and Martínez-Marcos 2003). EOG recordings demonstrate that both the olfactory and vomeronasal epithelia respond to pheromones in female red-bellied newts (*C. pyrrhogaster*) and male red-spotted newts (*N. viridescens*), although the largest responses are recorded in the VNO (Park and Propper 2002; Toyoda et al. 1999). These results suggest that the functions of the olfactory organ and VNO overlap, and that a specific organ may not be exclusively linked to a specific suite of behaviors.

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