



Terrestrial toadlets use chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour

PHILLIP G. BYRNE* & J. SCOTT KEOGH†

*School of Biological Sciences, Monash University

†School of Botany and Zoology, Australian National University

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Among anuran amphibians evidence for chemical communication is scarce. We carried out three experiments to evaluate whether chemosignals influence the sexual behaviour of an Australian terrestrial toadlet, *Pseudophryne bibronii*. Substrate choice trials (experiment 1) revealed that females preferred to associate with substrate marked by either sex rather than an unmarked substrate and that males preferred substrate marked by females, but avoided substrate marked by other males. These results suggest that the odour of both sexes functions as a sexual attractant and that male odour may also function to repel potential male competitors. In experiment 2 we assessed whether females use male chemosignals during mate location by making gravid females navigate a two-choice Y-maze to reach calling males. Almost invariably, females followed a path outlined with male gland secretions. This result indicates that male chemosignals combined with acoustic signals improve the ability of females to find nest sites. In experiment 3 we tested whether conspecific odour influences the calling behaviour of nesting males. Female odour stimulated a twofold increase in advertisement calling and male odour stimulated a switch to territorial calling. These findings indicate that nesting males use conspecific odour as a cue for regulating investment in acoustic courtship and territory defence. Our results advance a small body of evidence to suggest that anurans use chemosignals to identify and locate potential mates and provide the first demonstration that odour can influence anuran calling behaviour.

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Many animals release odorous chemical compounds (chemosignals) that allow individuals to recognize members of the same species, identify their gender and ascertain reproductive status (Wyatt 2003). Consequently, chemosignals are often important for sexual communication and reproduction. Within sexes, chemosignals can provide information that is used to mediate competition for access to the opposite sex or the control of vital resources (e.g. nest sites). These chemosignals may operate directly by signalling dominance and regulating contests (Moore et al. 1997) or indirectly by defining territory boundaries (Mathis 1990; Gosling & Roberts 2001). Between sexes, chemosignals can facilitate sexual union by advertising when individuals are ready to mate, attracting potential partners and

aiding in mate localization (Drickamer 1999; Sorensen & Stacey 1999). Once the sexes are together, chemosignals can also strongly influence whether copulation takes place. Such effects can often be pronounced, for example when one sex (usually females) releases a chemosignal that triggers the opposite sex (usually males) to perform elaborate visual or acoustic courtship displays (Wyatt 2003). The dependency of animals on chemosignals to mediate social interactions is taxonomically widespread with more aspects of animal behaviour being influenced by chemosignals than any other sensory mode (Wyatt 2003). Nevertheless, for several animal classes it remains to be determined whether chemical signalling is important for sexual communication. More importantly, we still have much to learn about the way chemosignals integrate with other sensory modes.

For amphibians, it is well known that urodeles (salamanders and newts) use chemosignals to regulate social hierarchies and coordinate sexual reproduction (Dawley 1998). In contrast, virtually nothing is known about chemical communication in anurans (frogs and toads). Anurans

Correspondence: P. G. Byrne, School of Biological Sciences, Monash University, VIC 3800, Australia (email: phillip.byrne@anu.edu.au). J. S. Keogh is at the School of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia.

produce conspicuous acoustic signals so it is generally assumed that this is the primary mode of communication (Gerhardt & Huber 2002). However, in many species males also possess breeding glands that release odorous peptides (Thomas et al. 1993; Smith et al. 2003) so chemically mediated sexual interactions could be widespread. Support for this notion comes from the demonstration that male chemosignals act as powerful mate attractants in two taxonomically and ecologically distinct frog species, the Australian magnificent treefrog, *Litoria splendida* (Wabnitz et al. 1999) and the aquatic dwarf African clawed frog, *Hymenochirus* sp. (Pearl et al. 2000). In addition, an archaic Leiopelmatid frog from New Zealand (*Leiopelma hamiltoni*) can recognize substrates that have been scent marked by conspecifics (Waldman & Bishop 2004). This frog does not vocalize and is the only surviving representative of a lineage that predates modern frog families. Therefore, there is good reason to suspect that chemical communication might have been the primary mode of anuran communication before acoustic signalling evolved. If this is this case, the capacity to communicate chemically might have been retained by a variety of anuran families. Clearly, whether chemosignals are commonly used by anurans to recognize and locate conspecifics is a question that requires empirical attention. More importantly, because most anurans rely on acoustic communication (Gerhardt & Huber 2002) it would be valuable to explore whether interactions occur between acoustic and chemical modes. There have been no previous attempts to test for such interactions.

Acoustic communication in anurans carries many costs, including high energetic expense, increased risk of predation (Ryan & Tuttle 1983) and an increased risk of dehydration (Judge & Brooks 2001), so in most species males call only intermittently (Gerhardt & Huber 2002). This constraint on acoustic signalling is often confounded by limited visual communication because males usually call at night and from cryptic locations (i.e. beneath vegetation; Gerhardt & Huber 2002). Under these restrictive conditions the use of chemosignals, which can be produced continuously and relatively inexpensively (Hedin et al. 1974), may complement acoustic signalling in a variety of ways. We consider two possibilities. First, chemosignals may help females localize calling males. If females are receptive to male chemosignals, in addition to their calls, they should be able to find mates more easily and reduce the multitude of costs (e.g. energetic expense and predation risk) that are generally associated with mate searching. Because chemosignals are much less directional than acoustic signals they may be of little use in long-range mate localization, but their capacity to provide long-lived messages could facilitate localization at close range (Wyatt 2003). The added benefit of using chemosignals would be that sexual communication could persist in situations where the acoustic system breaks down. Among anurans acoustic signalling will be interrupted when fluctuating costs of calling force males to call sporadically (see above), or when males advertise from concealed locations and their calls are diffused by physical barriers (Gerhardt & Huber 2002). Second, chemosignals may permit males to detect conspecifics and to adjust calling behaviour according to the risk of male–male competition (male

presence) or the chance of mate attraction (female presence). Anurans generally produce different call types to repel rivals (territorial calls) and attract mates (advertisement calls) and these calls are almost always more effective when produced at higher rates (Gerhardt & Huber 2002). To attract females over long distances males must invest at least some energy in calling. However, if males could restrict extreme call investment to periods when conspecifics are known to be in close proximity they could maximize their chance of attracting mates and/or repelling rivals while minimizing costs arising from unnecessary calling. Chemical cues are known to stimulate acoustic signalling in some insects (e.g. fruit flies: Antony et al. 1985) and a few vertebrate groups such as gekkonid lizards (Regaldo 2003) and mice (Holy & Guo 2005) but there has been no attempt to test for this association in anurans.

The Australian terrestrial toadlet *Pseudophryne bibronii* provides a prime opportunity to investigate chemical communication by an anuran amphibian. The species communicates acoustically but also has pronounced dorsal, axillary and postfemoral glands that exude highly odorous mucus during the breeding season. The ecology of *P. bibronii* is similar to that of salamanders that use chemical communication whereby breeding takes place at night under thick layers of leaf litter and visual contact between conspecifics is restricted. In this environment it seems likely that chemosignals will be used in conjunction with acoustic signals to mediate territoriality, courtship and mating (Mitchell 2005). Our aim in this investigation was to explore whether *P. bibronii* use chemical cues to communicate sexual information. We addressed this aim by carrying out three manipulative experiments. In the first experiment we focused on whether toadlets can recognize conspecific chemosignals by running a series of substrate choice trials. Assuming that odour functions as a mate attractant, and possibly also as a territorial repellent, we predicted a similar pattern to that found in dwarf African clawed frogs and magnificent treefrogs where individuals respond positively to chemosignals produced by the opposite sex but not by the same sex (Wabnitz et al. 1999; Pearl et al. 2000). In the second experiment we focused on whether chemosignals help females localize acoustically advertising males. We predicted that if females rely on chemosignals even when acoustic signals are available they would bias their movement towards a trail of male gland secretions. In the third experiment we examined whether chemosignals released by conspecifics affect the calling behaviour of resident males. In the field, male *P. bibronii* are known to adjust their calling behaviour in response to the presence of silent conspecifics (unpublished data) so we predicted that conspecific chemosignals would stimulate a similar response.

METHODS

Study Species

Pseudophryne bibronii is a small myobatrachid toadlet (22–36 mm snout–vent) that is endemic to temperate regions of southeastern Australia. The species is a terrestrial breeder with oviposition taking place in shallow nests that are constructed and defended by males. The nests are

typically found in moist soil underneath logs, rocks or dense leaf litter in low-lying, seasonally inundated areas (Pengilley 1973; Woodruff 1977). Males establish nests in autumn, following the first heavy rain, and then remain with them for 3–5 months. Over this period males produce two distinct call types which serve to advertise territory occupation and attract mates, hereafter referred to as the 'territorial call' and the 'advertisement call', respectively. Females move into the breeding site several weeks after the males and they visit several nests before choosing a mate (unpublished data). During mating, males grasp females in front of their hindlegs (inguinal amplexus) and fertilize eggs externally as they are released into the nest. The embryos, which are individually surrounded by jelly capsules, develop up to Gosner stage 26–27 at which point development is suspended until the nest is flooded by heavy winter rainfall and hypoxia triggers tadpoles to hatch into temporary pools (Bradford & Seymour 1985, 1988a,b). Tadpoles remain in these pools until they metamorphose in spring or early summer when the pools begin to dry (Woodruff 1977).

Study Site

The study took place in a natural breeding population located in remnant *Eucalyptus*, *Banksia* and *Casuarina* forest near Wrights Beach in Jervis Bay National Park, New South Wales, Australia. Male toadlets were nesting in soil burrows underneath leaf litter along a drainage line (60 m long × 2–3 m wide) that flowed into a semipermanent creek (Stony Creek). The drainage line was naturally dry during the study but flooded in the following winter months. We collected data between 1800 and 0600 hours (Australian Eastern Standard Time) from 24 March to 18 June 2005.

Capture and Transport

We caught toadlets with drift fences and pit traps that enclosed the breeding site. Traps were checked every 2–3 h after nightfall and a final check was made after dawn to ensure that no toadlets remained trapped during the day. At the bottom of every pit trap we placed moist sand and leaf litter so that the toadlets remained hydrated and received protection from the elements as well as potential predators. Once the toadlets were collected we took two measures to avoid mixing chemosignals produced by different individuals. First, every time new animals or experimental equipment was handled we wore fresh disposable, plastic, surgical gloves. Second, when we transported toadlets around the study site, or between the study site and the field station, we kept individuals in separate, ventilated, plastic Ziplock bags. Each bag contained moist tissue so that the toadlets did not dehydrate. The bags were disposed of after every use. Every toadlet that was captured was photographed so that we could identify individuals by their unique vent patterns and ensure that individuals were never used in more than one experimental trial.

The study was conducted with permission of the Australian National University Animal Experimentation

Ethics Committee and the New South Wales National Parks and Wildlife Service.

Experiment 1: Substrate Recognition

Toadlets of both sexes were collected from the study site and returned to a field station (approximately 1 km away) where they were housed individually in clear plastic boxes (22 × 15 cm and 10 cm high) lined with moist tissue paper that permitted cutaneous water uptake. To avoid chemical contamination, no food was offered. Boxes were stored in a quiet room and kept under conditions of ambient light and temperature. On the following night the tissue (weight = 10 g) was removed, rolled into a ball, and placed in one corner of a new plastic 'test box' (22 × 15 cm and 10 cm high). In the opposite corner of the test box was placed moist 'blank' tissue paper (weight = 10 g) that did not contain toadlet chemosignals. We then collected a new male or female from the study site and brought it back to the field station where it was stored in a clear plastic storage box (22 × 15 cm and 10 cm high) lined with moist tissue and left to acclimate in complete darkness for 1 h.

After this time we staged choice trials at ambient temperature by placing the test toadlet in the centre of the test box containing the two tissue paper types, one situated to the left and the other to the right. We reversed the position of the two tissue paper types every trial to avoid any corner effects. Each trial consisted of a test toadlet being left undisturbed in a test box in a darkened room at ambient temperature (19–24°C) for 15 min, after which time we noted its position. If the toadlet was within 2 cm of the tissue paper we scored it as receptive to substrate marked by conspecifics, but if it was more than 2 cm away it was scored as unreceptive to the substrate. Using this design we tested the preferences of each sex against chemosignals from the same sex (versus a blank) and the opposite sex (versus a blank). Each trial involved new toadlets so that each test toadlet received an odour stimulus from a unique individual. We ran 61 trials involving 62 males and 60 females. Trials were run on 23 nights over 6 weeks. Treatments were randomized across the experimental period. All test toadlets were returned to their exact site of capture no more than 2 h after the completion of a trial. Trials had no detectable impact upon the health of the toadlets. This observation was supported by the fact that most individuals were recaptured later in the season. Following each trial a test box was washed with 75% ethanol, followed by rainwater, to remove any residual chemical signals. To determine whether toadlets associated with tissue containing conspecific chemosignals more often than 'blank' tissue paper we used one-tailed binomial probability tests. We used one-tailed tests because a pilot study using the same experimental design, but with smaller sample sizes, indicated that toadlets preferred substrate marked by conspecifics (P. Byrne, unpublished data).

Experiment 2: Mate Location

To assess whether females use chemosignals during mate location we used a two-choice Y-shaped maze. The

maze was constructed of clear Plexiglas and consisted of a straight tube with an entrance hole (15 cm long \times 3 cm diameter) and two branching arms, each with an exit hole (each arm = 15 cm long \times 3 cm diameter). In one arm of the maze a sexually active male was gently placed against the Plexiglas and wiped back and forth for approximately 3 min so that his gland secretions formed a visible smear running from the main tube into the arm. We consider that this procedure did not provide unnaturally high concentrations of chemosignal because we removed only secretions that were covering the male's glands at the time of collection. The other arm of the maze was left untouched so that no chemosignal was available. The maze was then placed in front of a calling male situated between the two branching arms, and a gravid female was released into the base of the straight tube. Because every calling male directly faced the maze there was no bias in the angle that females received the acoustic signal. The purpose of the calling male was to provide an acoustic signal that would entice a female to move down the maze until she reached the branch point, where she would have to make a choice to move into either the 'chemical present' arm or the 'chemical absent' arm. We used only males that were calling at a low rate (3–5 calls/min) so that females would move slowly through the maze and have ample time to detect and process the chemosignal presented. This approach was based on the assumption that females would be more dependent on chemosignals for localization under conditions where acoustic signals were weak. Test females were scored as having made a choice only after they exited one of the arms. We ran 28 trials over 2 weeks. All trials were run between 1900 and 2400 hours when frogs are active. Every trial involved a new female and two new males (one to provide calls and one to provide secretions). In this way no toadlet was used twice. All males and females used in the trials were captured on site approximately 15 min prior to testing and were returned to the site of capture immediately after trial completion. No trial lasted more than 30 min. Between every trial the Y-shaped maze was washed with 75% ethanol, followed by rainwater, to remove residual chemicals. We also alternated placement of the chemical cue between the right and left arms of the maze to avoid any 'side' effects. To determine whether the proportion of females that chose an arm with the chemosignal was significantly different from 50% we used a two-tailed binomial probability test. We used a two-tailed test because we had no a priori reason to expect that female toadlets would use chemosignals during mate location.

Experiment 3: Male Call Behaviour

We recorded the calls of nesting 'resident' males before and after presenting them with either a male or female that we had wrapped in tissue paper. Using this technique we allowed nesting males to receive chemosignals in natural concentrations but blocked the presence of visual and tactile signals. Audio signals were also unavailable because test toadlets remained silent during trials. We ensured this was the case by importing audio recordings into Canary 1.2.4 (Cornell Laboratory of Ornithology, Ithaca, NY,

U.S.A.) and examining oscillograms for sound production (frequency range examined = 100 Hz–10 kHz). During trials test toadlets were positioned approximately 5 cm away from the nest of a 'resident' male. All test toadlets were caught at the breeding site and were used in trials within 5 min of capture. They were returned to their exact site of capture immediately after trials were completed. No trial lasted more than 25 min. We also ran a control treatment where we presented resident males with tissue paper that did not contain a test toadlet. This treatment was a control for the possibility that the presence of tissue, or disturbance during tissue placement, influenced the call behaviour of a resident male. We recorded calls for 3 min before and 3 min after treatment. All recordings were made with a Sharp MDLP mini-disc recorder and a Sennheiser ME66 microphone (Sennheiser, Wedemark, Germany). Prior to each trial, air temperature was measured ($\pm 0.1^\circ\text{C}$) using a Miller and Webber thermometer. Measurements were taken at ground level approximately 5 cm away from a male's nest site. We ran 45 trials comprising 15 replicates for each of the three treatments. New resident males and test toadlets were used for each trial so that each toadlet was used only once and data points were independent. The experiment was run over 3 consecutive nights and every trial involved independent toadlets.

From the recordings, we counted the calls produced by resident males and categorized them as 'advertisement' calls or 'territorial' calls. Differences before and after treatment in (1) call rate (average number of calls/min) and (2) call type (number of advertisement calls/(number of advertisement calls + number of territorial calls)) were independently compared using ANCOVA. For analysis the effects were treatments (male present, female present or control) and the dependent variables were either the change in call rate after – before or the change in call type (after – before) and the covariate was air temperature. Air temperature was included as a covariate because it can influence call behaviour in anurans (Gerhardt & Huber 2002).

RESULTS

Experiment 1: Substrate Recognition

Female toadlets preferred to associate with male-marked substrate rather than a blank substrate in 12 of 13 trials, which was significantly more than expected (one-tailed binomial test: $P < 0.01$). Females also preferred female-marked substrate to the blank more often than expected (12/16 trials; $P < 0.05$). Male toadlets preferred female-marked tissue paper to the blank more often than expected (13/15 trials, $P < 0.05$), but did not choose male-marked tissue rather than the blank more than expected (10/17 trials, NS).

Experiment 2: Mate Location

All of the females tested moved through the Y-maze but 22 of 28 females moved into the arm that contained male chemosignals. This was significantly more than expected by chance (two-tailed binomial test: $P < 0.01$).

Experiment 3: Male Call Behaviour

Male call rate (calls/min) was significantly influenced by experimental treatment but not air temperature (ANCOVA: treatment: $F_{2,44} = 36.59$, $P < 0.001$; air temperature: $F_{1,44} = 0.038$, NS). Males increased their call rate in response to the presence of chemosignals released by both males and females but this increase was more than twice as large in response to female chemosignals. The presentation of blank tissue paper (control) resulted in a negligible (<1%) increase in call rate (Table 1).

Male call type (proportion of advertisement versus territorial calls) was also influenced by experimental treatment, but not by air temperature (ANCOVA: treatment: $F_{2,44} = 120.53$, $P < 0.001$; air temperature: $F_{1,44} = 0.015$, NS). Resident males increased territorial calls by approximately 18% in response to female chemosignals but by more than 70% in response to male chemosignals (Table 2). The blank tissue paper control resulted in only a slight increase (<1%) in the proportion of territorial calls produced.

DISCUSSION

The combined results of our three experiments provide convincing evidence that the Australian terrestrial toadlet *P. bibronii* uses chemosignals to recognize conspecifics, locate mates and strategically adjust calling behaviour. We discuss the results of each experiment in turn. The substrate choice experiment (experiment 1) showed that toadlets preferred substrates previously occupied and marked by other toadlets to unmarked substrates. This result indicates that toadlets preferred environments with odour to those without odour and provides evidence that toadlets have the ability to recognize conspecifics based on their scent. However, there were sexual differences in preference patterns. While females preferred substrate marked by either sex, males only preferred substrate marked by females and avoided substrate marked by other males. The result that both males and females preferred substrate marked by the opposite sex was predicted because in many animals, including two frog species (magnificent treefrogs and dwarf African clawed frogs), chemosignals function as mate attractants (Wabnitz et al. 1999; Pearl et al. 2000; Wyatt 2003).

Chemical mate attractants have probably evolved in toadlets because of constraints on alternative modes of sexual communication. The efficacy of visual signalling is impaired because breeding takes place at night and in

Table 1. Call rate per min ($\bar{X} \pm SE$) of nesting male terrestrial toadlets before and after nest site disturbance (control), or the presentation of male or gravid female odour at the nest site

	Before	After	After–before
Control	8.66±0.606	8.93±0.752	0.26±0.671
Male	8.66±0.694	12.86±0.861	4.20±0.603
Female	9.20±0.725	17.73±1.216	8.53±0.613

For each treatment there were 15 replicate trials.

Table 2. Proportion of advertisement calls ($\bar{X} \pm SE$) produced by resident male terrestrial toadlets before and after nest site disturbance (control), or the presentation of a male or gravid female odour

	Before	After	After–before
Control	0.993±0.006	0.986±0.013	0.006±0.035
Male	0.973±0.015	0.272±0.035	0.700±0.031
Female	0.980±0.010	0.801±0.041	0.178±0.032

For each treatment there were 15 replicate trials.

locations hidden under leaf litter (Mitchell 2005). There is evidence from studies in other vertebrates that impaired visual communication favours the use of chemosignals. For example, comparative analysis of communication modes in grysbok antelope, *Raphicerus melanotis*, has shown that species living in densely vegetated areas are more dependent on chemical communication than species inhabiting open spaces (Albone 1984). In toadlets, acoustic signals are also unreliable because when the soil in the nest site is dry males refrain from calling to avoid dehydration (Mitchell 2001). Under these conditions of restricted visual and acoustic communication, chemical attractants are likely to benefit both sexes by increasing the likelihood of sexual union (see below).

The result that females preferred to associate with female-marked substrate was unexpected and contrasts with a study for dwarf African clawed frogs that found females were unresponsive to chemosignals produced by other females (Pearl et al. 2000). A possible explanation for female–female attraction in toadlets is that females reduce costs associated with mate searching (e.g. energetic expense and risk of dehydration) by using the scent trails of other females to locate nest sites. Costs of mate searching are likely to be particularly high in toadlets because females are polyandrous (mate with multiple males) and spend extended periods (sometimes weeks) moving between nest sites (Pengilly 1973; Woodruff 1977). Females may also be attracted to female chemosignals because it benefits them to aggregate. For instance, by following conspecific pheromones females could aggregate in communal hiding places that provide protection (Parzefall et al. 2000).

The finding that males avoided male-marked substrate suggests that male chemosignals may have a repellent function. This effect has not been reported previously in frogs but is known to occur in some newts where males produce a pheromone that repels rivals during intrasexual mating competition (Park & Propper 2001). A repellent function for male odour would directly benefit resident toadlets because a male's reproductive success hinges on his ability to secure a nest site with soil moisture levels that fall within a narrow range required for offspring development (Bradford & Seymour 1988a, b). Although nesting males can effectively ward off rivals by using territorial calls, this method of defence can be unreliable because calling activity fluctuates with soil moisture and hydration state (Mitchell 2001). Chemosignals probably offer a more consistent means of advertising nest occupation and provide a primary defence against territory take-over. However, not all of the males we tested avoided male-marked substrate. One explanation for this variance is that territory

intruders use the concentration of chemosignals to assess the competitive ability of resident males and are deterred only if a signal represents a significant threat (Lee & Waldman 2002). If it does not, intruders may be encouraged to attempt a take-over. Such context-dependent agonistic behaviour occurs in territorial salamanders where males respond aggressively to the odour of smaller males but submissively to that of larger males (Mathis 1990).

The two-choice Y-maze experiment (experiment 2) showed that when approaching the nest site of a calling male, females almost invariably followed a path outlined by male secretions. This result provides evidence that chemosignals assist females during close-range mate localization. The benefit of our experimental design was that it allowed us to assess whether chemical cues are used in situations where acoustic cues are also available. Playback experiments have shown that female toadlets can locate speakers broadcasting male advertisement calls, which indicates that acoustic cues alone are sufficient for mate localization (unpublished data). Therefore, our finding that females followed a chemical trail, even when acoustic signals were available, shows that toadlets use a dual system of mate attraction/localization. It is not uncommon for behaviours to be mediated by multisensory pathways but this is particularly likely in species where individuals are challenged by a diverse ecological environment (Ganzhorn 1990). As mentioned above, breeding in toadlets takes place at night under cryptic conditions. In this environment, visual signals will be poorly transmitted but both acoustic and chemical signals should remain effective because they can convey information in the dark and permeate physical barriers such as leaf litter (Wyatt 2003), although acoustic signals broadcast from behind barriers are also susceptible to diffusion (Gerhardt & Huber 2002). By combining acoustic and chemical signals toadlets may increase the efficacy of message transfer. For example, in the desert ant *Aphaenogaster albisetosus* scouts that have discovered a food source can more effectively alert other individuals to join them if they combine pheromone and sound signals (Hölldobler & Wilson 1990). Alternatively, the use of a dual system may have evolved as a redundancy mechanism to overcome limitations inherent in both acoustic and chemical systems and increase the chance that a message is delivered. For instance, although acoustic signals can be transmitted quickly they are expensive to produce and provide only short-lived messages. In contrast, chemosignals can be produced cheaply (Hedin et al. 1974) and provide long-lasting messages but they have the disadvantages of being transmitted slowly and providing unreliable sources of directional information (Wyatt 2003). Since we did not test whether chemosignals influence female behaviour in a context independent from acoustic signals, it remains to be determined whether chemosignals are sufficient for mate localization when they are the only mode of information transfer.

In experiment 3, we allowed nesting males to receive chemosignals from conspecifics but blocked them from receiving any visual, tactile or auditory stimuli. This treatment had a substantial impact upon male call behaviour. In response to male chemosignals residents switched to the production of territorial calls, whereas in response to female

chemosignals residents more than doubled their investment in advertisement calling. This result, that resident males responded differently to chemosignals from each sex, provides compelling evidence that toadlets can use odour to distinguish the gender of conspecifics. The finding that resident males switched to territorial calling in response to male odour indicates that chemosignals alerted them to an increased risk of competition. By recognizing the presence of a concealed intruder, and responding aggressively, resident males are likely to pre-empt physical competition and increase their chance of retaining a nest site. Male chemosignals can elicit territorial behaviour in several vertebrates, including mammals (Holy & Guo 2005), reptiles (Regaldo 2003) and urodele amphibians (Mathis 1990) but our results provide the first demonstration that they can do so in anurans. Given that in many anuran species males defend resources required by females (e.g. oviposition sites; Heatwole & Sullivan 1995), chemically stimulated territorial calling could be a common adaptation.

The finding that female chemosignals stimulated a substantial increase in advertisement calling indicates that detection of female odour alerts males to an increased chance of mate attraction. Male mating success in *P. bibronii*, as for most other frogs, correlates positively with call effort so there is strong pressure on males to advertise persistently (Mitchell 2001; Gerhardt & Huber 2002). However, calling also carries substantial costs, including energetic expense and increased risk of dehydration (Mitchell 2001; Gerhardt & Huber 2002). Therefore, by being receptive to female chemosignals males may minimize the costs of advertisement while maximizing the probability of mating success. Females may also benefit from the increase in call rate if a more continuous acoustic signal expedites localization of nest sites and reduces search costs (see above). In many animal groups males are known to increase the intensity of acoustic courtship display in the presence of females (Gerhardt & Huber 2002; Patricelli et al. 2002; Holy & Guo 2005), but there are very few species where chemosignals are known to mediate this response. A classic example is the fruit fly *Drosophila melanogaster* where detection of female odour incites males to stridulate their wings and produce a song that is essential for successful courtship (Antony et al. 1985). Similar responses to female odour have also been found in vertebrates. In hemidactylus house geckos, *Hemidactylus mabouia*, males housed in female-scented cages increase the production of courtship calls (Regaldo 2003). Similarly, in laboratory mice, *Mus musculus*, the smell of female urine triggers males to produce ultrasonic vocalizations that appear to be used in courtship (Holy & Guo 2005). Although anurans have been a model group in the investigation of acoustic communication, our study is the first to provide evidence that calling activity is regulated in response to chemical cues. Therefore this result makes an important contribution to our understanding of the way anurans integrate sensory modes to cope with fluctuating social conditions. Future studies of acoustic communication in anurans should consider possible influences of chemosignals on behaviour.

Despite the potential advantages to anurans of using a dual system of acoustic and chemical communication, evidence that both modes are used in combination has

been obtained for only two other frog species, the Australian magnificent treefrog and the dwarf African clawed frog (Wabnitz et al. 1999; Pearl et al. 2000). Little is known about the breeding biology of the former so it is difficult to understand why chemosignals have been favoured in addition to acoustic signalling. In dwarf African clawed frogs breeding takes place at night in turbid water so it seems likely that, similar to *P. bibronii*, constraints on visual and acoustic signalling may have favoured the exploitation of chemical signalling. Many acoustically advertising anurans breed under visually restricted conditions (e.g. at night) and possess breeding glands that are structurally similar to those found in salamanders that are known to communicate chemically (Houck & Sever 1994). Therefore it seems unusual that there have not been more reported cases of anurans combining auditory and olfactory communication systems. In Hamilton's frog, *Leiopelma hamiltoni*, which is an ancient anuran lineage, acoustic communication is absent but chemicals play a fundamental role in social communication (Waldman & Bishop 2004). Therefore, breeding glands in modern anurans may simply be vestigial characters that reflect a dependence on chemosignals prior to the evolution of acoustic systems. An alternative and more likely scenario is that chemical communication is still widely used by anurans but researchers have overlooked its significance (Waldman & Bishop 2004). In support of the latter argument is the knowledge that many anuran species use chemicals for diverse purposes other than social communication, such as navigation (Sinsch 1990) and predator detection (Flowers & Graves 1997). This indicates that the anatomical and physiological attributes required for odour production and reception remain functional in many groups. Clearly, further studies testing for chemical communication are now required across a range of anuran species to determine whether chemosignals are commonly used to mediate sexual interactions.

In summary, this study provides evidence that the terrestrial toadlet *P. bibronii* uses chemicals to recognize conspecifics and to locate mates. Furthermore, the study provides novel evidence that chemosignals permit males to detect concealed conspecifics and make subsequent adjustments in calling behaviour according to the risk of male–male competition (male presence) or the chance of mate attraction (female presence). Terrestrial toadlets join a small but growing list of frogs known to communicate using a combination of auditory and chemical signals. Interactions between these sensory modes may be an important, but currently overlooked, system of regulating sexual interactions in anuran amphibians.

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