

## RESEARCH ARTICLE

# Male serrate-legged treefrogs adjust competition strategies according to visual or chemical cues from females

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### ABSTRACT

There is increasing evidence that many anurans use multimodal cues to detect, discriminate and/or locate conspecifics and thus modify their behaviors. To date, however, most studies have focused on the roles of multimodal cues in female choice or male–male interactions. In the present study, we conducted an experiment to investigate whether male serrate-legged small treefrogs (*Kurixalus odontotarsus*) used visual or chemical cues to detect females and thus altered their competition strategies in different calling contexts. Three acoustic stimuli (advertisement calls, aggressive calls and compound calls) were broadcast in a randomized order after a spontaneous period to focal males in one of four treatment groups: combined visual and chemical cues of a female, only chemical cues, only visual cues and a control (with no females). We recorded the vocal responses of the focal males during each 3 min period. Our results demonstrate that males reduce the total number of calls in response to the presence of females, regardless of how they perceived the females. In response to advertisement calls and compound calls, males that perceived females through chemical cues produced relatively fewer advertisement calls but more aggressive calls. In addition, they produced relatively more aggressive calls during the playback of aggressive calls. Taken together, our study suggests that male *K. odontotarsus* adjust their competition strategies according to the visual or chemical cues of potential mates and highlights the important role of multisensory cues in male frogs' perception of females.

**KEY WORDS:** Anurans, Competition strategy, *Kurixalus odontotarsus*, Multisensory cues, Social context

### INTRODUCTION

In many species, males compete directly for females (intrasexual selection) or enhance their attractiveness to females (intersexual selection) by increasing display rates, evolving ornaments or improving signal conspicuousness (Darwin, 1871; Kirkpatrick and Ryan, 1991; Searcy and Andersson, 1986). Generally, individuals adjust their competition strategies based on the immediate ecological context to reduce the risk of injury or death by predation, as well as the overall energy cost of advertising (Montiglio et al., 2017; Patricelli et al., 2002). In most anuran

species, males vocalize to attract females (Wells, 2007), and they commonly modify their calling behavior according to the social context to improve signal efficacy and consequently attractiveness. For instance, male serrate-legged small treefrogs (*Kurixalus odontotarsus*) produce calls with an increased maximum note number and increased ratio of notes to calls under highly competitive pressure (Zhu et al., 2017a). This strategy helps to minimize an individual's energy cost while maximizing benefit when competition is high (Zhu et al., 2017a). A study on African clawed frogs (*Xenopus laevis*) showed that receptive but not unreceptive calls from females increased the call duration of previously subordinate males, indicating that males altered their competition investment based on the receptivity conveyed by the females' calls (Xu et al., 2012).

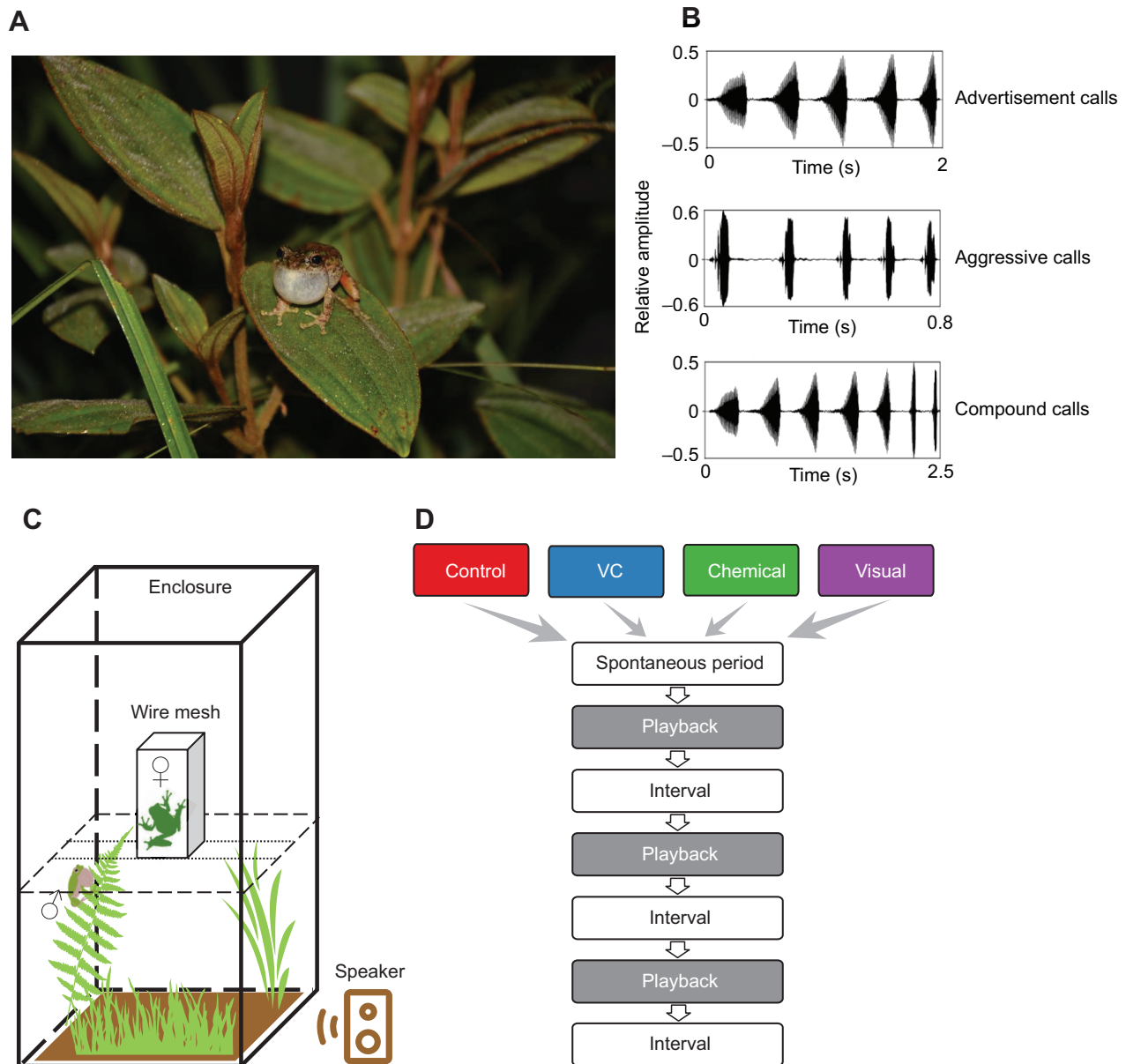
In addition to vocal signals, male courtship behavior may also contain visual, chemical or vibrational signals (Halfwerk et al., 2019; Stamberger et al., 2014). Females often improve their communication efficiency and/or accuracy and evaluate the potential mate by integrating multisensory signals from males, such as audio-visual cues (Stange et al., 2017; Taylor et al., 2007; Taylor and Ryan, 2013) and audio-vibratory cues (Halfwerk et al., 2017). In contrast, multimodal cues or alternative cues may also affect male–male interactions (Narins et al., 2018; Preininger et al., 2013b; Still et al., 2019). For example, a study with *Allobates femoralis* showed that only acoustic playback of vocalizations coupled with vocal sac pulsations could provoke physical attacks by a territorial male (Narins et al., 2003). Similarly, calling rates of male túngara frogs (*Physalaemus pustulosus*) significantly increased in response when ripples were added to sound playback (Halfwerk et al., 2014a). There is increasing evidence that many anurans use alternative cues to detect, discriminate and/or locate conspecifics (Halfwerk et al., 2014b; Narins et al., 2005; Preininger et al., 2013a); however, fewer studies have focused on how male frogs detect the presence of potential mates and whether males respond differently to a competitor in relation to the female cue detected.

Serrate-legged small treefrogs (*K. odontotarsus*) are tropical anurans are distributed in Yunnan, Guizhou, Guangxi, Guangdong and Hainan in China, and the reproduction of this species occurs mainly from April to August (Fei et al., 2010; <https://amphibiansoftheworld.amnh.org/>). Generally, males vocalize on branches or in bushes to attract females (Fig. 1A). The calls of male *K. odontotarsus* comprise two types of notes: a wideband A note and a narrowband B note (Zhu et al., 2017c). Males commonly produce three kinds of calls with these two notes: (1) advertisement calls, which contain a series of A notes (e.g. 5A, Fig. 1B), (2) aggressive calls, which contain a series of B notes (e.g. 5B, Fig. 1B), and (3) compound calls, which contain a series of A notes followed by several B notes (e.g. 5A2B, Fig. 1B). Zhu et al. (2017b) reported that advertisement calls and compound calls are attractive to female frogs. Once they enter the amplexus, females leave the leks to find a suitable place to lay their eggs (Zhu et al., 2016). In

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**Fig. 1. Study animal, acoustic stimuli, apparatus and procedures used in this study.** (A) A calling male *Kurixalus odontotarsus* (photo by K.D.). (B) Oscilloscope recordings of three acoustic stimuli. Top: advertisement calls 5A; middle: aggressive calls 5B; bottom: compound calls 5A2B (Zhu et al., 2017c). (C) Individual males were placed in testing enclosures constructed of wire mesh ( $42 \times 32 \times 90 \text{ cm}^3$ ). A female was placed in a wire-mesh cage ( $12 \times 14 \times 28 \text{ cm}^3$ ) or a cylindrical opaque glass jar (12 cm in diameter  $\times$  20 cm in height) that was in the central area of the male testing enclosure. Soil and plants were provided (drawing by K.D.). (D) In each treatment group, 5A, 5B and 5A2B were broadcast in a randomized order after a spontaneous period, and each period lasted for 3 min. VC represents the visual and chemical group.

addition, during fieldwork, special odors can be perceived in both males and females of the species, especially when individuals are handled (field observations). These characteristics make *K. odontotarsus* a suitable model species for investigating the effects of multisensory cues on male–male competition, which have not been examined in this species.

In the present study, we examined whether male *K. odontotarsus* use visual or chemical cues to detect females and thus alter their competition strategies. Our previous study has demonstrated that advertisement calls and compound calls (i.e. calls containing A notes) elicit an increased calling rate and induce males to produce an increased number of B notes and total number of notes (Zhu et al., 2017b). In contrast, aggressive calls (i.e. calls containing only B notes) suppress responses, and a decreased number of A notes and

total number of notes were produced (Zhu et al., 2017b). In addition, males produce more compound calls under higher competitive pressure (Zhu et al., 2017a). To examine whether and how the presence of females affect male–male competition strategies in different calling contexts, three acoustic stimuli (advertisement calls, aggressive calls and compound calls) were broadcast in a randomized order after a spontaneous period to focal males in one of four treatment groups: visual and chemical cues from a female, only chemical cues from a female, only visual cues from a female, and a control (with no females). We predicted that males would produce a greater number of advertisement calls and compound calls in response to the presence of females to enhance attractiveness and consequently mating success. We also predicted that the calling investment would be highest during exposure to multisensory cues.

## MATERIALS AND METHODS

### Study site and subjects

Our study was conducted from May to August 2019 at Diaoluo Mountain National Nature Reserve in Hainan, China (18.72°N, 109.87°E, elevation 933 m). The experiments were carried out between 20:00 h and 01:00 h under natural light. The average temperature was  $20.3 \pm 0.1^\circ\text{C}$ , and the average relative humidity was  $93.7 \pm 0.3\%$ .

Three to five gravid female and 6–8 calling male *Kurixalus odontotarsus* (Lv et al., 2018) were collected from the chorus per night. Frogs were housed individually in plastic cages ( $28 \times 18 \times 15 \text{ cm}^3$ ) with soil and plants before the tests. Animals were kept in the cages for less than 3 h and were returned to the chorus after the tests. Experiments were conducted in the field, at least 300 m away from the collection site. Individual males were placed in testing enclosures constructed of wire mesh ( $42 \times 32 \times 90 \text{ cm}^3$ ), which were open to ambient air and sound (Fig. 1C). The enclosures were placed in sites that were far enough from the chorus to prevent the tested males from directly interacting with other males. Soil and plants were provided, and male frogs could locomote freely in enclosures (Fig. 1C).

### Experimental procedures

To investigate how the presence of females affected male–male competition strategies and whether males responded differently in relation to the female cue detected, each male was assigned to one of the following four treatment groups: (1) combined visual and chemical cues from a female (VC), (2) only chemical cues, (3) only visual cues and (4) a control (with no females). In the VC group, a gravid female was placed in a wire-mesh cage ( $12 \times 14 \times 28 \text{ cm}^3$ ) that was in the central area of the male testing enclosure. The cage was supported by two iron bars (Fig. 1C). Therefore, the two individuals could easily interact with each other, but they could not engage in amplexus. In the chemical group, the wire-mesh cage was covered with a thin layer of black cloth to block visual cues, but the chemical cues of the females were accessible through the cloth. In the visual group, we replaced the wire-mesh cage with a cylindrical opaque glass jar (12 cm in diameter  $\times$  20 cm in height) with moss and plants so that the chemical cues of the females were inaccessible, but the visual cues were retained. Females were handled gently by the same person, and allowed to acclimate for at least 10 min before each trial. We used different females in each trial. Although the dorsal colors of females were different, it was not a variable to consider because their colorations were similar to the background in the natural habitat.

We recorded males for 3 min before initiating a playback (i.e. spontaneous period), 3 min during the playback of a specific stimulus, and 3 min after the playback (Fig. 1D) in one of the treatment groups using a digital voice recorder that was equipped with internal microphones (Sony PCM-D100). Three acoustic stimuli (advertisement calls, 5A; aggressive calls, 5B; compound calls, 5A2B; Fig. 1B) were broadcast in a randomized order using a speaker (amplified field speaker, Saul Mineroff Electronics, Inc.), which was 1 m away from the enclosure. Stimuli were presented using Adobe Audition (version 3.0) and broadcast at a peak level of 80 dB SPL (re. 20  $\mu\text{Pa}$ ) (Zhu et al., 2017b), measured using a sound pressure level meter (AWA 6291, Hangzhou Aihua Instruments Co., China).

We recorded the latency to the male's first call. Males who failed to vocalize within 15 min after being placed in the enclosure were excluded from the study and were returned to the chorus. In addition, we recorded the total number of notes, total number of calls, notes/calls, number of compound calls, number of advertisement calls, number of aggressive calls, total number of A notes, total number of B notes, maximum number of A notes and

maximum number of B notes during each 3 min period, which were obtained using Adobe Audition 3.0 software (California, USA). After testing, the male frogs were measured (snout–vent length, SVL), weighed and marked with a toe-clip prior to release at the original capture location to prevent recapture ( $N=189$ ;  $\text{SVL}=32.22 \pm 0.12 \text{ mm}$ ;  $\text{mass}=2.01 \pm 0.02 \text{ g}$ ;  $\text{means} \pm \text{s.e.m.}$ ). Marking was approved by the management office of the Mt Diaoluo Nature Reserve and the Animal Care and Use Committee of the Chengdu Institute of Biology, CAS (CIB2016042403).

### Statistical analysis

All statistical tests were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). The total number of notes and total number of calls, number of advertisement calls and total number of A notes and maximum number of A notes, number of aggressive calls and total number of B notes and maximum number of B notes were highly correlated in all calling contexts (Spearman correlation analysis, all  $P < 0.05$ , Table S1). Therefore, the total number of notes, notes/calls, total number of A notes, maximum number of A notes, total number of B notes and maximum number of B notes were excluded in subsequent analysis.

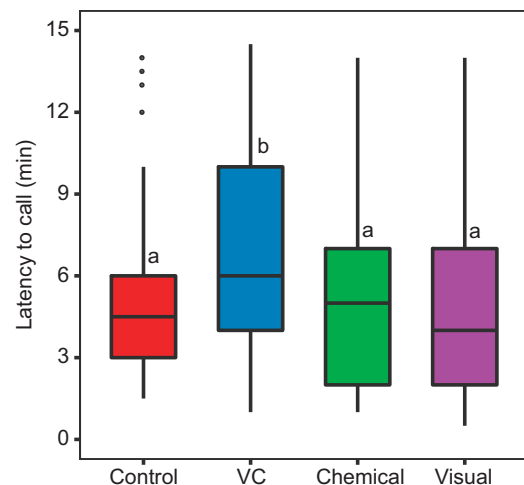
Data were examined using the Shapiro–Wilk test to determine their normality, and not all data indicated a normal distribution ( $P < 0.001$ ). Consequently, to examine whether and how the presence of females affected male–male competition strategies in a specific calling context, we used the Kruskal–Wallis test to detect the difference among the treatment groups and used a two-sided Mann–Whitney *U*-test to determine the differences between pairs of groups.  $P < 0.05$  was considered statistically significant.

## RESULTS

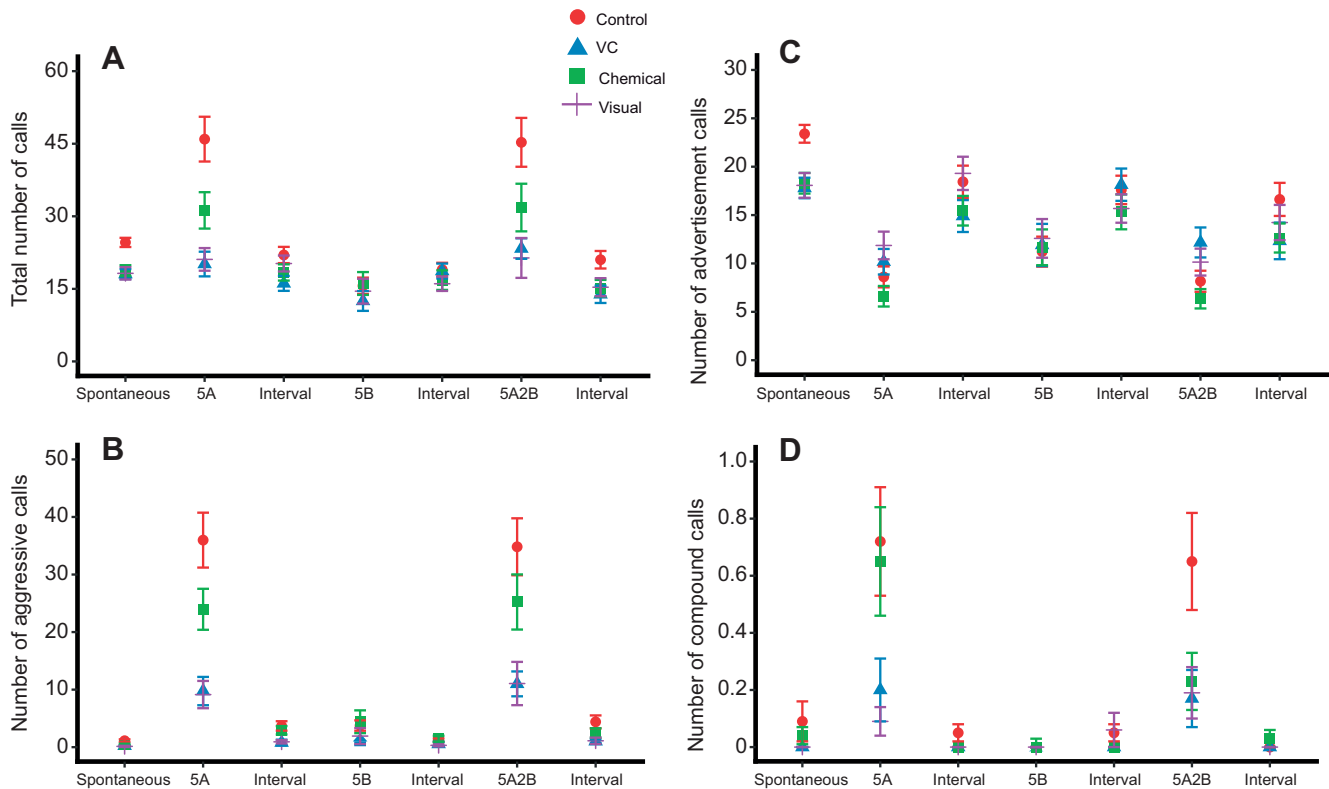
A total of 189 male *K. odontotarsus* were included in this study, but only 179, 141 and 178 males responded to stimuli 5A, 5B and 5A2B, respectively. The latency to call in the VC group was longer than values recorded in the other three groups ( $\chi^2=10.124$ , d.f.=3,  $P=0.018$ , Fig. 2).

### Evoked vocal responses in males

Stimuli 5A and 5A2B induced males to produce a greater total number of calls (Fig. 3A) and number of aggressive calls



**Fig. 2. Latency to call in male *K. odontotarsus*.** Call latency was measured in control group ( $N=58$ ), visual and chemical group (VC,  $N=41$ ), chemical group ( $N=48$ ) and visual group ( $N=42$ ). Different superscript letters indicate significant differences ( $P < 0.05$ ) as determined by the Mann–Whitney *U*-test.



**Fig. 3. The total number of calls and number of different call types in male *K. odontotarsus*.** Total number of calls (A), number of aggressive calls (B), number of advertisement calls (C) and number of compound calls (D) during the spontaneous period ( $N=189$ ), stimulus playback periods (5A:  $N=179$ ; 5B:  $N=141$ ; 5A2B:  $N=178$ ) and intervals (5A interval:  $N=147$ ; 5B interval:  $N=139$ ; 5A2B interval:  $N=151$ ). Each period lasted 3 min.

(Fig. 3B) but fewer advertisement calls (Fig. 3C) than those produced during the spontaneous period in all treatment groups. In contrast, stimulus 5B induced males to produce fewer total number of calls (Fig. 3A) and number of advertisement calls (Fig. 3C) than those produced during the spontaneous period in all treatment groups. Only during the playback of advertisement calls and compound calls, did males in all treatment groups produce compound calls (Fig. 3D).

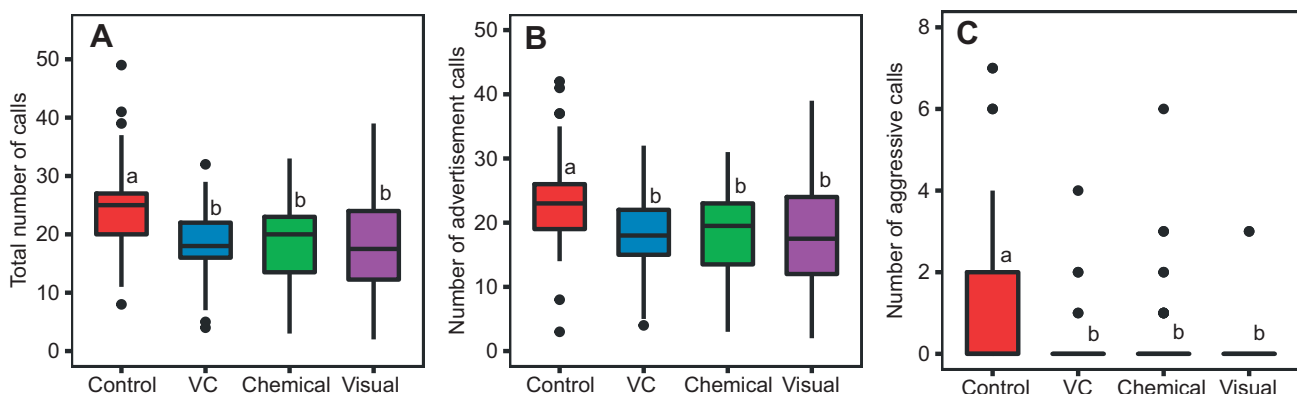
#### The effects of female stimuli on male–male competition strategy in different calling contexts

During the spontaneous period, males in the VC group, chemical group and visual group produced fewer total number of calls ( $\chi^2=23.427$ ,

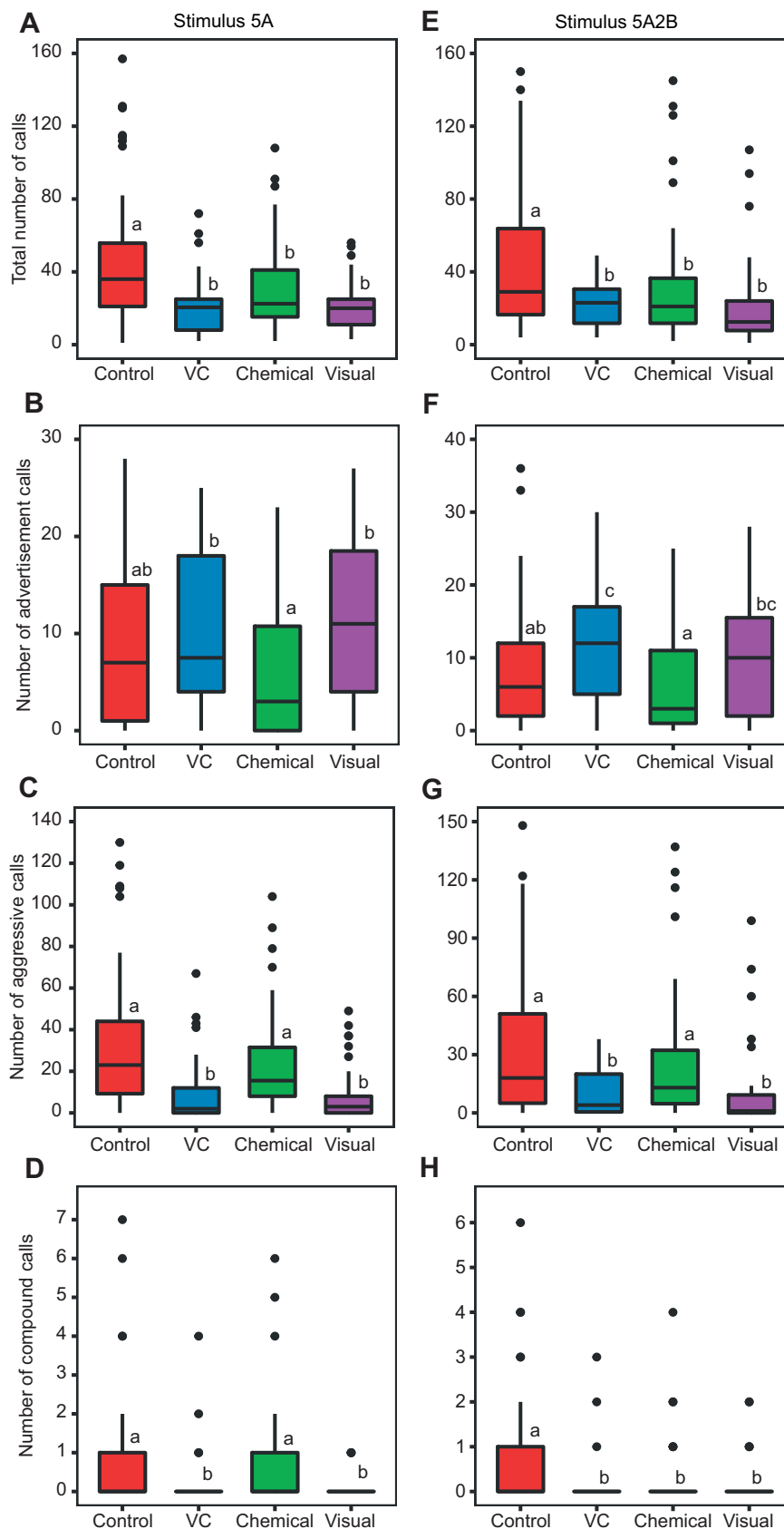
$d.f.=3$ ,  $P<0.001$ , Fig. 4A), advertisement calls ( $\chi^2=17.764$ ,  $d.f.=3$ ,  $P<0.001$ , Fig. 4B) and aggressive calls ( $\chi^2=22.348$ ,  $d.f.=3$ ,  $P<0.001$ , Fig. 4C) than those in the control group.

During the playback of advertisement calls (5A), males in the VC, chemical group and visual group produced significantly fewer total number of calls ( $\chi^2=24.268$ ,  $d.f.=3$ ,  $P<0.001$ , Fig. 5A) than those in the control group. Males in the VC group and visual group produced significantly more advertisement calls ( $\chi^2=9.645$ ,  $d.f.=3$ ,  $P=0.022$ , Fig. 5B) but fewer aggressive calls ( $\chi^2=44.366$ ,  $d.f.=3$ ,  $P<0.001$ , Fig. 5C) and compound calls ( $\chi^2=14.910$ ,  $d.f.=3$ ,  $P=0.002$ , Fig. 5D) than those in the chemical group.

During the playback of compound calls (5A2B), males in all treatment groups produced significantly fewer total number of



**Fig. 4. Comparison of different calls during the spontaneous period.** Calls were compared among the control group ( $N=58$ ), VC group ( $N=41$ ), chemical group ( $N=48$ ) and visual group ( $N=42$ ). (A) Total number of calls. (B) Number of advertisement calls. (C) Number of aggressive calls. Different superscript letters indicate significant differences ( $P<0.05$ ) as determined by the Mann–Whitney *U*-test.



**Fig. 5. Comparison of different calls in response to stimulus 5A or stimulus 5A2B.** Calls were recorded in the control group ( $N=58$ ), VC group (5A:  $N=40$ ; 5A2B:  $N=36$ ), chemical group (5A:  $N=46$ ; 5A2B:  $N=48$ ) and visual group (5A:  $N=35$ ; 5A2B:  $N=36$ ). (A,E) Total number of calls. (B,F) Number of advertisement calls. (C,G) Number of aggressive calls. (D,H) Number of compound calls. Different superscript letters indicate significant differences ( $P<0.05$ ) as determined by the Mann–Whitney  $U$ -test.

calls than those in the control group ( $\chi^2=17.089$ , d.f.=3,  $P=0.001$ , Fig. 5E). Males in the VC and visual group produced significantly more advertisement calls ( $\chi^2=10.032$ , d.f.=3,

$P=0.018$ , Fig. 5F) but fewer aggressive calls ( $\chi^2=29.825$ , d.f.=3,  $P<0.001$ , Fig. 5G) than those in the chemical group. In addition, males in all treatment groups produced significantly



fewer compound calls than those in the control group ( $\chi^2=10.782$ , d.f.=3,  $P=0.013$ , Fig. 5H).

During the playback of aggressive calls (5B), the total number of calls (Fig. 6A) and number of advertisement calls (Fig. 6B) did not vary significantly across treatments. Males in the VC group and visual group produced significantly fewer aggressive calls ( $\chi^2=14.268$ , d.f.=3,  $P=0.003$ , Fig. 6C) than those in the chemical group.

## DISCUSSION

The importance of multimodal cues in intersexual selection has been well studied with respect to female choice, but whether different cues from females elicit a different response in males has received less attention. In the present study, we conducted an experiment to investigate the effects of visual and chemical cues from females on male–male interactions in *K. odontotarsus*.

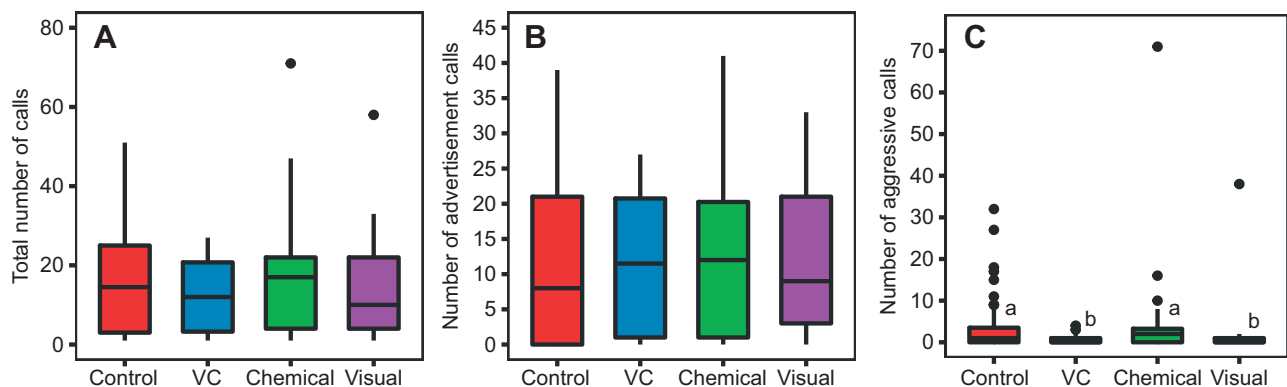
First, we found that the playback of advertisement calls (5A) and compound calls (5A2B) elicited more responses and induced focal males to produce more aggressive calls than those produced during the spontaneous period in all treatment groups. In contrast, playback of aggressive calls (5B) suppressed responses, and focal males produced fewer advertisement calls than those produced during the spontaneous period, regardless of the treatment. These findings are in substantial agreement with those of Zhu et al. (2017b), who suggested that stimuli containing A notes elicit vocal responses, whereas stimuli containing only B notes suppress vocal responses. Our results further demonstrate that the behavioral response of male *K. odontotarsus* to a specific type of call is highly repeatable.

In addition, we found that males would not produce aggressive calls independently in the presence of any stimuli from females. Zhu et al. (2017b) reported that the B note itself was not sufficient to elicit phonotaxis from females. These results confirm two types of functional components in mating calls of *K. odontotarsus* and suggest that B notes are functional for males only. This is consistent with what has been found in other anuran species. For example, male túngara frogs tend to produce only whines when they call alone but add chucks to the whines during vocal competitions (Ryan et al., 2019). On the other hand, the chuck enhances the attractiveness of the whine, but the chuck itself does not elicit attraction of the females (Ryan et al., 2019).

Contrary to expectations, comparisons among groups showed that males reduced the total number of calls in response to the presence of females during the spontaneous period and the playback of advertisement calls and compound calls. Numerous studies have

suggested that male competitiveness increases in the presence of potential mates (Aspbury and Gabor, 2004; Cox and Le Boeuf, 1977; Miranda et al., 2015; Montgomerie and Thornhill, 1989; Xu et al., 2012). For example, female calls of the Emei music frog (*Nidirana daunchina*) not only incite male–male physical contact or nest plundering but also elicit an increased number of aggressive calls and advertisement calls (Cui et al., 2010). A study on beetles (*Librodor japonicus*) also showed that the intensity of male fighting escalated in the presence of females (Okada and Miyatake, 2004). Presumably, the presence of a female increases the probability of mating in winners under these circumstances. In contrast, a study on leafhoppers (*Aphrodes makarovi*) suggested that a female responded equally to advertisement calls emitted by both winners and losers, and mated with the first male that located her, regardless of his investment in calling (Kuhelj et al., 2017). Usually, a female frog initiates courtship by approaching a calling male, and the male initiates amplexus by climbing on the back of the female (Gramapurohit et al., 2011; Montanarin et al., 2011; Ovaska and Rand, 2001). Therefore, once a female is nearby, it is possible that locating the female is more productive than continued calling. In addition, males may decrease their energy expenditure and predation risk by reducing costly unnecessary calling.

Comparisons between pairs of groups showed that males in the chemical group produced relatively fewer advertisement calls but more aggressive calls than those in the VC group and visual group during the playback of advertisement calls and compound calls. In contrast, males in chemical group produced relatively more aggressive calls than those in the VC group and visual group during the playback of aggressive calls. Forester and Thompson (1998) reported that male American toads (*Anaxyrus americanus*) were attracted to female-derived odors, demonstrating the role of chemical cues in mate localization. Similarly, a study on Australian toadlets (*Pseudophryne bibronii*) suggested that chemical cues aid individuals in close-range mate location and identification (Byrne and Keogh, 2007). In the present study, we found that the behavioral responses of male *K. odontotarsus* to specific acoustic stimuli varied according to the way that females were perceived. There are many studies that have demonstrated that frogs can discriminate familiar and unfamiliar individuals based on the advertisement calls (Bee, 2016); however, it is unknown whether frogs could discriminate males and females based on odors. Therefore, it is possible that although males can utilize chemical cues alone to detect females, they may perceive the invisible female frog combined with the acoustic playback as a rival male.



**Fig. 6.** Comparison of different calls in response to stimulus 5B. Calls were recorded among the control group ( $N=56$ ), VC group ( $N=22$ ), chemical group ( $N=36$ ) and visual group ( $N=27$ ). (A) Total number of calls. (B) Number of advertisement calls. (C) Number of aggressive calls. Different superscript letters indicate significant differences ( $P<0.05$ ) as determined by the Mann–Whitney *U*-test.

Consequently, males produced more aggressive calls in response to advertisement calls when the visual cues were unavailable. In addition, we found that the latency to call in the VC group was significantly longer than those in the other groups, suggesting that a combination of visual and chemical cues may help male frogs to enhance detectability and identification of potential mates.

To date, chemical communication in anurans has been overlooked in contrast with other taxa (Woodley, 2015). Our findings provide a new example of airborne chemical cues that could help males detect females. Recent studies have suggested that chemical cues are likely used to identify species (Starnberger et al., 2013) and even to discriminate sex (Brunetti et al., 2019) in treefrogs. Further study is needed to identify the components of female-derived secretions and to investigate the mechanism of perception in *K. odontotarsus*. Overall, the present study suggests that male *K. odontotarsus* adjust their competition strategies according to the presence of females and highlights the important roles of visual and chemical cues in male frogs' perception of females.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: K.D., J.-G.C.; Methodology: K.D., Q.-L.H., B.-C.Z., J.-G.C.; Software: Q.-L.H.; Validation: J.-G.C.; Formal analysis: K.D.; Investigation: K.D., Q.-L.H., Y.Z., T.-L.W., J.-C.W.; Resources: T.-L.W., J.-C.W., J.-G.C.; Data curation: K.D., Q.-L.H., Y.Z.; Writing - original draft: K.D.; Writing - review & editing: K.D., B.-C.Z., J.-G.C.; Visualization: K.D.; Supervision: J.-G.C.; Project administration: J.-G.C.; Funding acquisition: K.D., J.-G.C.

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#### Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.229245.supplemental>

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