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# Parental Care and Acoustic Communication of the Smooth Guardian Frog *Limnonectes palavanensis*, a Bornean Frog With Possible Sex-Role Reversal

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Parental Care and Acoustic Communication of the Smooth Guardian Frog *Limnonectes palavanensis*, a Bornean Frog With Possible Sex-Role Reversal

Johana Goyes Vallejos, PhD

University of Connecticut, 2016

Sexual selection theory predicts that the sex contributing most toward the viability of the offspring will become the choosy sex. In most animal species, females have higher parental investment; thus, sexual selection typically acts more strongly on males, making the females choosy. On rare occasions, male parental investment is so high that it limits the potential for additional mating opportunities. In these cases, females compete for males and males become the choosy sex, leading to a sex-role reversed system. The sex-role reversal hypothesis states that males invest more in the offspring than females do, females display sexually selected traits more intensely than males, and females outnumber males in the mating pool, leading to more intense intrasexual competition among females. I characterized the vocal repertoire and parental care behaviors in the smooth guardian frog *Limnonectes palavanensis* in Brunei Darussalam (Borneo Island) in order to test predictions of the sex-role reversal hypothesis. I found that males perform all parental duties, attending the eggs for 9–11 days, and then transporting the tadpoles on their backs to a suitable deposition site. These deposition sites are scarce, which may increase the number of days it takes a male to return to the mating pool. Choice experiments testing deposition site preferences demonstrated that males do not avoid predators or conspecific tadpoles. In addition, males may split their tadpoles among nearby pools. Moreover, I described the vocal repertoire of male and female *L. palavanensis*. Males exhibit an advertisement call and a courtship call not previously described for this species. Remarkably, I found that females gather around a calling male and start calling spontaneously at higher rates than those of the males, a behavior not previously reported in anurans. Using playback stimuli, I found that males

do not defend territories and lack an aggressive call, however, they exhibit male-male acoustic interference. Females increase their calling rate when a simulated male is present but there was no evidence that they respond differently to female calls. The prolonged male parental care behavior and the calling behavior of *L. palavanensis* constitute evidence for sex-role reversal in this species.

Parental Care and Acoustic Communication of the Smooth Guardian Frog *Limnonectes palavanensis*, a Bornean Frog With Possible Sex-Role Reversal

Johana Goyes Vallejos

B.Sc., Universidad del Valle, Cali – Colombia, 2016

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Parental Care and Acoustic Communication of the Smooth Guardian Frog *Limnonectes palavanensis*, a Bornean Frog With Possible Sex-Role Reversal

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*A mis Padres y Hermano,  
los pulmones de mi vida  
y mi razón para continuar*

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## CHAPTER 1. INTRODUCTION

Females are the choosy sex in a variety of animal mating systems. Males compete for females through direct male-male combat or through elaborate displays. The strength of sexual selection acting on each sex is determined by each sex's relative parental investment and which sex becomes the limiting resource for the opposite sex. If male parental care investment is costly and limits the availability of males, females will compete for males and males can become the choosy sex (Trivers 1972). This is known as sex-role reversal. Evidence for sex-role reversal has been observed in sea horses and pipefishes, belostomatid bugs, jacanas, and phalaropes (Smith 1979, Reynolds 1987, Vincent et al. 1992, Emlen et al. 1998).

One of the most studied vertebrate examples of sex-role reversal is the family Syngnathidae. Laboratory and field studies have shown that several species of pipefishes and some species of seahorses are completely or partially sex-role reversed. In these species, males carry the eggs in a special pouch where they take care of them until they hatch (Berglund et al. 1986). In most populations, females are more abundant than males and they compete with each other for access to the males. Thus, when the operational sex-ratio is female biased, males become the choosy sex, usually choosing larger females (Berglund 1993, 1995). In some species, sex roles in courtship change seasonally as the sex ratio becomes biased toward females late in the breeding season. Males with specialized brood pouches have a limited amount of space to carry the eggs, and they have to care for them for a long period of time. On the contrary, females usually lay more than one clutch with more than one male, which means that the reproductive rate exceeds that of the males (Berglund and Rosenqvist 1993). The behavioral characteristics of these species are consistent with the sex-role reversal hypothesis.



In anurans, the poison frog *Dendrobates auratus* was thought to present some degree of sex-role reversal, because males invest in parental care and there is female-female competition (Wells 1978). However, males compete with each other for mating opportunities as well. Thus, even though females take an active role in courtship, and also compete aggressively with other females for access to males, it is not a case of complete sex-role reversal (Summers 1989). Other anurans show some features of sex-role reversal. In the Majorcan midwife toad (*Alytes muletensis*), males call from hidden locations that are hard for females to locate, and females often call to males to elicit a courtship call that gives information about the male's location. Females also engage in aggressive physical competition, but males do so as well (Bush and Bell 1997). In the Chinese concave-eared torrent frog (*Odorrana tormota*), both males and females call in very noisy environments. Males exhibit precise phonotaxis toward female calls, the reverse of the usual pattern in frogs (Shen et al. 2008).

Generally, to demonstrate the occurrence of sex-role reversal, it is expected that males invest heavily in parental care and receptive females should outnumber receptive males, so males become a limiting resource for females. In addition, males should behave as the choosy sex, since they are investing more per mating event. Likewise, more than one female should approach a male for mating opportunities and show some degree of acoustic or physical competition for access to males (Trivers 1972, Wells 2007).

### *Study System*

The smooth guardian frog (*Limnonectes palavanensis* Boulenger 1894) belongs to the family Dicroglossidae, a clade of Southeast Asian frogs. Some species within the clade have unusual sexual characters. For example, the males are larger than females, the reverse of the

usual pattern in frogs, and males bear small bony odontoid processes (fangs) along with hypertrophied jaw muscles, which are used in male-male aggressive competition (Emerson et al. 2000). *Limnonectes palavanensis*, a small nocturnal leaf litter frog found in rainforests on the Island of Borneo and in the Palawan Islands of the Philippines, exhibits unusual breeding behaviors compared with the other members of the family. In this species, males are smaller than the females and do not have fangs. Males give an advertisement call from the leaf litter on the forest floor to attract females. Inger et al. (1988) described the parental care behavior of *L. palavanensis* when they found males guarding the eggs and transporting tadpoles to water. Despite these descriptions, nothing is known about the time a male invests in taking care of the eggs until they hatch, and details of the tadpole transport behavior are still lacking. The calling repertoire has never been described, and to the best of my knowledge, female calling in this species has never been reported.

### *Objectives*

With its male parental care and female calling, *L. palavanensis* offers a unique opportunity to test predictions of sexual selection theory. For my dissertation research, I studied the parental care and vocal behavior of *L. palavanensis* and generated predictions of behavior that is expected under the sex-role reversal hypothesis: (1) Male parental investment is high, (2) tadpole deposition sites are rare, thereby increasing the “time-out” of the males for mating, (3) males are dispersed in space and do not hold territories, and male–male competition is low, and (4) female mate search costs are high, female-female competition is high, and more than one female should approach a male for mating opportunities.

In Chapter 2, I describe the parental care behavior of males, including male guarding behavior, the time required for tadpoles to hatch, and the process of how tadpoles climb on the back of the male.

In Chapter 3, I describe the tadpole deposition behavior of males in the field. Suitable natural tadpole deposition sites were identified and checked over five months for the presence of tadpoles. I also placed artificial pools throughout my study area to determine what environmental variables affect the decision of a male to deposit his tadpoles in a specific pool. I also designed a paired choice experiment to determine if males prefer to deposit their offspring in artificial pools free of predators or conspecifics.

In Chapter 4, I describe the vocal repertoire of male and female *L. palavanensis* based on observations in the field. Males produce advertisement calls and courtship calls, and females give a short courtship call. I quantified spectral and temporal properties of the three types of calls found in this species and determined the context in which these calls are produced. Females are highly vocal, often calling spontaneously even in the absence of males. I recorded events in which more than one female was found calling around a male and estimated the proportion of these events compared with occasions in which only one male and one female were found interacting with each other. I also quantified male and female calling rates throughout the night over several months to test the prediction from the sex-role reversal hypothesis that females vocalize more intensely than males.

In Chapter 5, I did playback experiments by broadcasting male and female calls to both males and females. I used the results of these playbacks to determine whether males are territorial (not expected under the sex-role reversal hypothesis) and whether females use vocal responses to female calls as aggressive signals (expected under the sex-role reversal hypothesis).

I tested whether males respond aggressively to a playback of a male advertisement call followed by a simulated female response. I also tested the behavior of males when presented with a playback of a female call followed by a male courtship call, a female call alone, and a male courtship call alone. Lastly, I tested if females compete acoustically among themselves. I first quantified changes in the calling behavior of the females when they were presented with a male advertisement with and without a simulated female response. Then, I estimated whether the number of calls produced by a female changes when presented with a playback of the male courtship call, compared to a playback of a female call followed by a male courtship call, simulating the presence of another female. If complete sex-role reversal occurs in this species, one would expect more vocal competition among females than among males, especially if another female is detected interacting with a receptive male.

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## CHAPTER 2. PARENTAL BEHAVIOR BY MALES OF THE SMOOTH GUARDIAN FROG

### *LIMNONECTES PALAVANENSIS* (BOULENGER 1894)

#### Introduction

Anuran amphibians exhibit a great diversity of reproductive modes. Currently, 40 different modes have been described (Haddad and Prado 2005, Wells 2007, Iskandar et al. 2014). Some of these are associated with some form of parental care (Crump 1996). Parental care in anurans can be male-only, female-only, or biparental, and includes egg attendance, egg transport, tadpole attendance, tadpole transport, froglet transport, and tadpole feeding (Wells 2007). Egg attendance is by far the most common mode of parental care, with the majority of cases occurring in the tropics. Although parental care has been more extensively studied in the tropical forests of Central and South America, in the Old World, attendance of terrestrial eggs has been observed in several families including bufonids, hemisotids, hyperoliids, and petropedetids in Africa (Amiet 1989, Mcdiarmid and Gorzula 1989, Amiet 1991), Passmore and Carruthers 1995), mantellids in Madagascar (Lehtinen 2003, Vences and de la Riva 2005, Wolf 2013), leiopelmatids in New Zealand (Bell 1985), most microhylids in Papua New Guinea (Bickford 2002, Bickford 2004), one species of rhacophorid in mainland Southeast Asia (Sheridan and Ocock 2008), and myobatrachids in Australia (Cogger 1992). Benefits of parental care include keeping the eggs moisten (Towsend et al. 1984, Burrowes 2000, Delia et al. 2013, Poo and Bickford 2013), reducing developmental abnormalities and fungal infection by jostling the eggs (Simon 1983), and protection against predators (Bickford 2004).

Most studies of anuran species with parental care have focused on the members of the Dendrobatidae, an anuran family known for exclusively exhibiting attendance of terrestrial eggs and tadpole transport either by males or females (Grant 2005). Tadpole transport on the back of a parent from a terrestrial nest to water is much less common. In fact, Noble (1927, p. 104) noted that “no other Salientia have breeding habits exactly like *Dendrobates* and *Phylllobates*”. However, egg attendance and tadpole transport similar to that of dendrobatids has been observed in two dicroglossid frogs of Borneo, *Limnonectes palavanensis* and *Limnonectes finchi*. Information on the parental behavior in these species is largely anecdotal (Inger et al. 1986, Inger and Voris 1988).

In this study, we report field and laboratory observations of egg attendance and tadpole transport in a population of the smooth guardian frog (*L. palavanensis*) in Brunei Darussalam. The smooth guardian frog is found throughout the northern part of Borneo, including Brunei Darussalam, the Malaysian state of Sarawak, a part of western Kalimantan, Indonesia, and the Palawan Island on the Philippines. However, it is likely that these populations actually represent a complex of species. The rough guardian frog (*L. finchi*) is restricted to the eastern half of Sabah, Malaysia. Both species inhabit the leaf litter of dipterocarp forests, where males call infrequently from widely separated locations. Females of *L. palavanensis* call in response to male vocalizations and often vocalize spontaneously sometimes in the absence of a calling male, forming small groups of two or three individuals to which the males respond with a short courtship call (Goyes-Vallejos unpublished data). Males give an advertisement call to attract females, mated pairs lay eggs on land, and the male subsequently attends the eggs and transports the tadpoles on his back to water, where they complete their development. Inger et al. (1986)

reported the first observation of frogs carrying tadpoles in Borneo. Later, Inger and Voris (1988) briefly described the parental behavior of *L. finchi* and *L. palavanensis* in Danum Valley Field Centre (N 5°19' E 119° 55') in Sabah, Malaysia. They observed males attending eggs and transporting tadpoles. They also found tadpoles in small shallow pools in intermittent streams, a shallow buttress tank, in a water-filled hole in a log, and in an animal wallow. Despite these descriptions, other aspects of the natural history of these species, such as clutch size, time the parent stays with the clutch, and the process by which the tadpoles climb onto the back of the parent remain unknown. The purpose of this study was to provide additional observations on the reproductive and parental behavior of *L. palavanensis*.

## **Methods**

### *Study site*

We studied the reproductive and parental behavior of *L. palavanensis* at the Kuala Belalong Field Studies Centre (KBFSC), Temburong district in Brunei Darussalam (115° 09' E, 4° 33' N and 50 – 200 m above sea level) from June to July 2012, July to November 2013, and June to December 2014; most of the observations for this study were made in 2014. The yearly rainfall at the site varies between 4900 and 6800 mm, with no distinct dry season (KBFSC Weather Data 2005 – 2014). The field station is located in the heart of the Ulu Temburong National Park. The vegetation around the field station is composed of dipterocarp primary forest and low-density understory vegetation. The topography is steep, with soils composed of sedimentary rocks and clay and a



shallow layer of leaf litter. For this study, we present the data from the 2013 and 2014 field seasons.

#### *Egg attendance in the laboratory*

From August to December 2014, we conducted acoustic encounter surveys (AES) in the area adjacent to the field station (ca. 1 ha) looking for calling males and females. Surveys were done from 1700h until 2300h using three main transects within the study area. When a female or a male were found, we took snout-urostyle measurements with a caliper (to the nearest 0.1 mm) and body mass using a digital portable scale (to the nearest 0.1 g). Both males and females were marked with a 4-digit identification number by clipping a toe for each hand and each foot. They were brought to the outdoor area of the lab and put together (one female and one male) in a glass terrarium (40x30x25 cm) with a plastic mesh cover and left alone overnight. The terraria were provided with a layer of clay soil, leaf litter and small invertebrates found in the forest to simulate the frogs' natural habitat. The terraria were sprayed with water every day to maintain 90% humidity inside, measured daily with a digital hygrometer. The next morning, if a clutch was found, we removed the non-attending adult, identified the sex of the attending adult using the toe-clip number, counted the number of eggs, and initiated behavioral observations of the parent. The non-attending adults were released at night at the point of capture.

Every 6 hours we observed the clutches for 5 minutes and recorded whether the attending parent was with the egg clutch (yes/no). We recorded the observations every day until the eggs hatched or the clutch was abandoned. We calculated the proportion of

observations the adult was attending the clutch. For some individuals, we used focal animal sampling *ad libitum* (constant observation up to 5 hours) using a Sony HDR-XR550V Handycam HD with the 'NightShot' setting to record in absolute darkness. We include descriptions of the hatching and climbing behavior of the tadpoles. Immediately after hatching, the parent with all the tadpoles on its back was returned to the point of capture.

### *Tadpole transport in the field*

During the 2014 field season, we visited the study area almost every night from 1700h to 2300h looking for adult *L. palavanensis* transporting tadpoles. When an individual was found, we approached it carefully and determined the number of tadpoles by photographing the back of the frog. In addition, we also photographed the front and hind feet to determine if the individual had been previously marked. We recorded the time at night and location. Individuals carrying tadpoles are very easily disturbed and are more sensitive to threats than are non-carrying individuals. The slightest disturbance makes them flee very quickly, so it was not possible to follow the tadpole-carriers to determine either the distance traveled or the tadpole deposition location.

## **Results**

### *Clutch size and egg attendance behavior*

On November 23, 2013, we found an adult male in the field attending a clutch of eggs at 2000h. The clutch had 14 eggs and it was in the exact spot where a female had been calling nine days before. The eggs were on top of a leaf and the males was covering

the eggs mass with his body and hiding under the leaf. The clutch hatched at around 0000h, but we did not observe when the tadpoles climbed on the back of the parent. This was the only observation of a male attending the eggs in the field in three field seasons.

Females and males hide deep in the leaf litter, so amplexus, fertilization, and egg deposition were never observed. Nine clutches of eggs were produced in terraria from August 21 to December 3, 2014. Eggs were deposited in leaf litter, and in all instances, the caregiving parent was a male. The mean  $\pm$ SD number of eggs per clutch was  $15 \pm 4$  (range: 10–21 eggs,  $n = 9$ ). Measurements of snout-urostyle length ranged from 26.0–28.6 mm for males and 29.0–33.0 mm for females. There was no correlation between number of eggs and female body size ( $P = 0.3$ ) or male body size ( $P = 0.4$ ). Hatching occurred  $10 \pm 1$  days (range: 9–11 days,  $n = 7$ ) after oviposition, between 1500h and 0000h. Tadpole size at hatching was very small, measuring 2.61–2.73 mm from the snout to the base of the tail, and a total length of 11–13 mm ( $n = 11$ , one clutch). The maximum width of the body was 2.72–2.84 mm. Tadpoles hatched at Gosner stages 24–25 (Gosner 1960), and their guts were full of yolk reserves, with the ventral intestinal coil beginning to form 24 hours after hatching.

We considered egg attendance as any behavior when the male was covering the clutch partially or completely, or when he was less than 1 cm away from the clutch (Fig 2.1). In 89% of the sampling observations (9 clutches, 352 observations), the male was attending the egg clutch (range: 81–98%). Nearly all of the observations when the male was not with the clutch were due to previous disturbances from the observers when the male had been attending the eggs. In one case, the male escaped when we were trying to remove the female. We put him back in the terrarium, but he did not return to the clutch

and was not observed attending the clutch at any time. However, the clutch of 14 eggs continued its normal development and after nine days, 11 tadpoles broke free of their egg capsules, two appeared to be unfertilized and the one stopped its development (Fig 2.2). In one instance, the male spent only 47% of the observations with the clutch of eggs. This particular clutch had 13 eggs, but from the day of oviposition, the eggs had been deposited in a scattered fashion and did not develop well. By day 11, most of the eggs looked decomposed and covered in fungus. At dusk, the male returned to the clutch site and retrieved two surviving tadpoles. One other clutch apparently was not fertilized and it was never observed to have a male attending it. Three complete clutches had all the eggs developed into tadpoles. Overall, 76% of the eggs laid in terraria developed into tadpoles (n = 9 clutches).

Approximately 24 hours after fertilization the eggs undergo rapid division with a clear differentiation of the vegetal pole and the animal pole (Fig 2.3A). After three days, neural folds have developed and fused to form the spinal cord and the brain (Gosner stage 15; Fig 2.3B). Head formation and tail elongation started four days after fertilization (Gosner stage 18–19 (tailbud stage); Fig 2.3C). When the embryos are six days old the external gills have formed and are already visible and eyes become apparent (Gosner stage 21–22; Fig 2.3D). By day nine, the eyes of the tadpoles are completely formed; their gut is full of yolk and they move inside the egg capsule when light is shined on them (Gosner stage 24–25; Fig 2.3E). After ten days the clutch is ready to hatch, the egg capsule seems to become thinner, and the tadpoles can break free (Fig 2.3F) (Gosner 1960).

During the continuous focal observations ( $n = 4$ ), attending males stayed with or near the clutch ( $< 1$  cm) during the whole observation period (sampling period = 50 min, 52 min, 85 min, 302 min, respectively). Males did not vocalize while attending a clutch of eggs, despite the fact that some females were calling in adjacent terraria. Attending males were observed feeding on small invertebrates only if the prey animals were crossing directly in front of them ( $n = 3$ ).

#### *Tadpoles climbing on males*

We observed tadpoles climbing on a male for three of the clutches in the laboratory (Fig 2.4). Even though tadpoles were capable of breaking free from the egg capsule, males apparently initiated physical stimulation of the eggs by gently touching the eggs with their fingers and their chins. They rotated and tapped the eggs, and the tadpoles responded by spinning inside the egg capsule. Subsequently, the male positioned himself on top of the eggs with the clutch under his abdomen, twitching his abdomen and fingers, and rotating his body on top of the clutch. He stepped several times on the eggs, separating them and breaking the egg capsule in the process. Once free, the tadpoles wriggled onto the back of the male while he remained still for several seconds. The male started a series of  $45^{\circ}$ – $90^{\circ}$  turns left to right or right to left while waiting for a few seconds after each turn for more tadpoles to climb onto his back. The tadpoles moved around on the back of the male continuously, rearranging themselves. Elapsed time from the moment the male stimulated hatching until the male left the oviposition site was 48, 50, and 87 min.

### *Tadpole transport in the field*

During 12 months of fieldwork, we found only 10 males transporting tadpoles (Fig 2.5). All of the observations occurred between 2000 and 2200 hours. In seven of these cases, it was possible to confirm the sex of the transporting adult as male, because it had been previously marked. The average number of tadpoles transported was  $13 \pm 3$  (range: 8–15). There was no correlation between body size and number of tadpoles ( $P = 0.2$ ,  $n = 7$ ). Since the frogs were already transporting tadpoles when discovered, it was not possible to determine the duration of tadpole transport.

We found one male with about 19 tadpoles on his back inside a pig wallow. The male had its hind limbs and fore limbs completely submerged and the tadpoles were in contact with the water (Fig 2.6). The tadpoles spun their tails several times before slowly dislodging from the back of the male. Not all of the tadpoles detached at once. Upon detaching, some of the tadpoles swam away, but others stayed underneath the male, which remained motionless for 7 min. Unfortunately, the male jumped away before depositing the rest of the offspring, and we were not able to find it again.

### **Discussion**

Our results confirmed earlier work by Inger and Voris (1988) that the caregiving parent in *L. palavanensis* is the male. Males of *L. palavanensis* exhibit prolonged attendance of eggs, staying with the eggs during the whole development period until hatching. Males did not leave the clutch for foraging or other activities, but did eat prey opportunistically if small invertebrates passed close to clutch. Males remain inactive during this time, presumably expending little energy. In a few instances, males were

observed shedding their skin and eating it. Therefore, the energetic cost of egg attendance may be minimal.

Males do not vocalize while attending the eggs, indicating that males do not attract additional females and that they take care of only one clutch at a time. This suggests that males are effectively forfeiting additional mating opportunities. In some species of dendrobatids in which the male guards the eggs and transport the tadpoles, males acquire additional mates, attending between two and four clutches (Brown et al. 2008, Roithmair 1994, Pröhl and Hödl 1999, Forti et al. 2013).

Observations of a clutch abandoned due to our disturbance of the male showed that, in the absence of predators, eggs can continue their normal development and can break free of the egg capsule without aid. However, without a male to retrieve and transport them to water, the aquatic tadpoles cannot complete development. Another of the clutches showed signs of poor development at the initial stages. This clutch had the lowest male attendance of all the observed clutches (47%). This indicates that males may assess the viability of the eggs and not allocate as much time to a defective clutch. In this particular case, only two tadpoles hatched out of thirteen eggs. The rest of the eggs were dull-looking, did not develop into embryos, and suffered fungal infection. It was remarkable, however, that the male returned to retrieve the two surviving tadpoles. We did not observe males of *L. palavanensis* eating unfertilized or infected eggs, nor did they exhibit disturbance-induced oophagy like some species of New Guinea microhylids exhibiting terrestrial egg attendance (Bickford 2004). In these species, males remove the fungal-infected eggs from the clutch to prevent further infection.

The number of tadpoles on the backs of males observed in the field is similar to the clutch sizes obtained in the lab, which suggests that males carry only one clutch at a time. Males retrieved all of the tadpoles in a clutch at once, a lengthy process that can take up to an hour and a half. In *Colostethus panamansis*, a dendrobatid frog of similar size, females were found carrying large number of tadpoles, suggesting that in this species the adult also carries all of the clutch at once (Wells 1980a), as observed in *L. palavanensis*. However, in other dendrobatid frogs such as *Oophaga pumilio* (Brust 1993), *Ranitomeya imitator*, *R. variabilis* (Brown et al. 2008), and *Allobates femoralis* (Ringler et al. 2013), the parent does not carry all the tadpoles simultaneously. Back space is not a limitation for males of *L. palavanensis* considering the small size of their tadpoles compared with the large size of the tadpoles relative to the parent size in the aforementioned species of dendrobatids.

Inger and Voris (1988) found males of *L. palavanensis* depositing tadpoles in pools of small intermittent streams and in puddles formed after rain, far away from any large body of water (i.e., permanent stream or river). Specifically, they observed tadpoles in a tree hole, in a pool on a fallen log, and in a small pool at the edge of the stream. These observations suggest that tadpole deposition sites are scarce and that males might have to spend a long time looking for these sites. The type of soil in our study site, coupled with the steep topography, does not allow for rain pools to last very long, rendering potential tadpole deposition sites ephemeral. In addition, tadpoles hatch with their guts full of yolk reserves, possibly allowing them to stay on the male for several days without feeding. In *C. panamansis*, tadpoles grow while sitting on the female's back, depending on their yolk reserves while the female transports them to water, a



process that can take up to nine days (Wells 1980b). Thus, tadpole transportation in *L. palavanensis* could last several additional days depending on the ability of the male to find tadpole deposition sites. This in turn would increase the reproductive ‘time out’ of the males and reduce the number of males available for mating.

Within *Limnonectes*, there is a wide spectrum of reproductive modes, from laying eggs in stream and ponds to internal fertilization and live birth of tadpoles (Tsuji 2004, Iskandar et al. 2014). Tadpole transport is unique to *L. finchi* and *L. palavanensis* and it has not been observed in any other species of frog in Southeast Asia. The most common mode of parental care in anurans is egg attendance (Wells 2007) and this is the case in a handful of species within *Limnonectes*. Egg attendance has been observed in *L. arathooni* (Brown and Iskandar 2000), *L. limborgi* (Rowley and Altig 2012), *L. finchi* (Inger et al. 1986), and *L. palavanensis* (Inger and Voris 1988, this study), but details about the egg attendance behavior are still lacking. Future research is needed to determine the function of egg attendance in *L. palavanensis* and the energetic and reproductive costs associated with it.

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Figure 2.1. Male of *Limnonectes palavanensis* attending a clutch of eggs. Photo by Johana Goyes-Vallejos.



Figure 2.2. **A.** Abandoned clutch of 14 eggs, note the formation of the animal pole (darkened area). **B.** The clutch continued its normal development (7 days old). Two eggs did not develop (yellow arrows) and one egg stopped developing after day five. The tadpoles hatched on their own after nine days. Photos by Johana Goyes Vallejos.

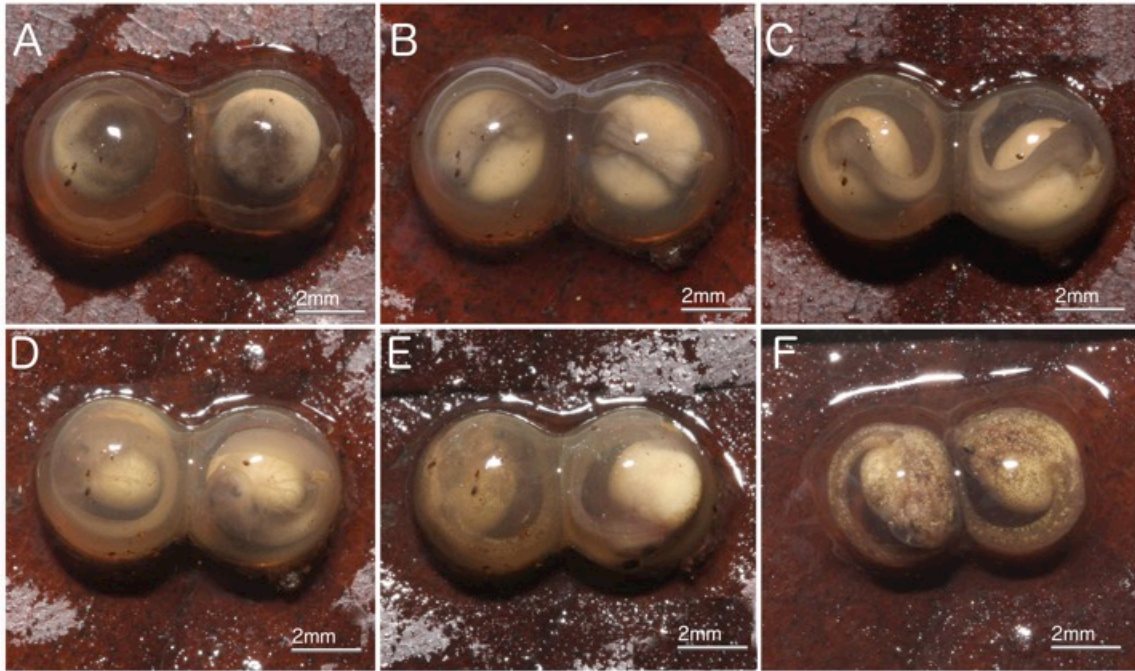


Figure 2.3. Embryonic development of the eggs of *L. palavanensis*. **A.** 24h after fertilization the vegetal pole differentiates from the animal pole. **B.** Neural folds develop, Gosner stage 15 (3 days old). **C.** The head forms and the tail elongates around the yolk sac, Gosner stage 18–19 (4 days old). **D.** Eyes become apparent and external gills are visible, Gosner stage 21–22 (6 days old). **E.** The tadpoles are now sensitive to light, the eyes are completely formed and the gills are not visible anymore, Gosner stage 24–25 (9 days old). **F.** The tadpoles are ready to hatch (10 days old).



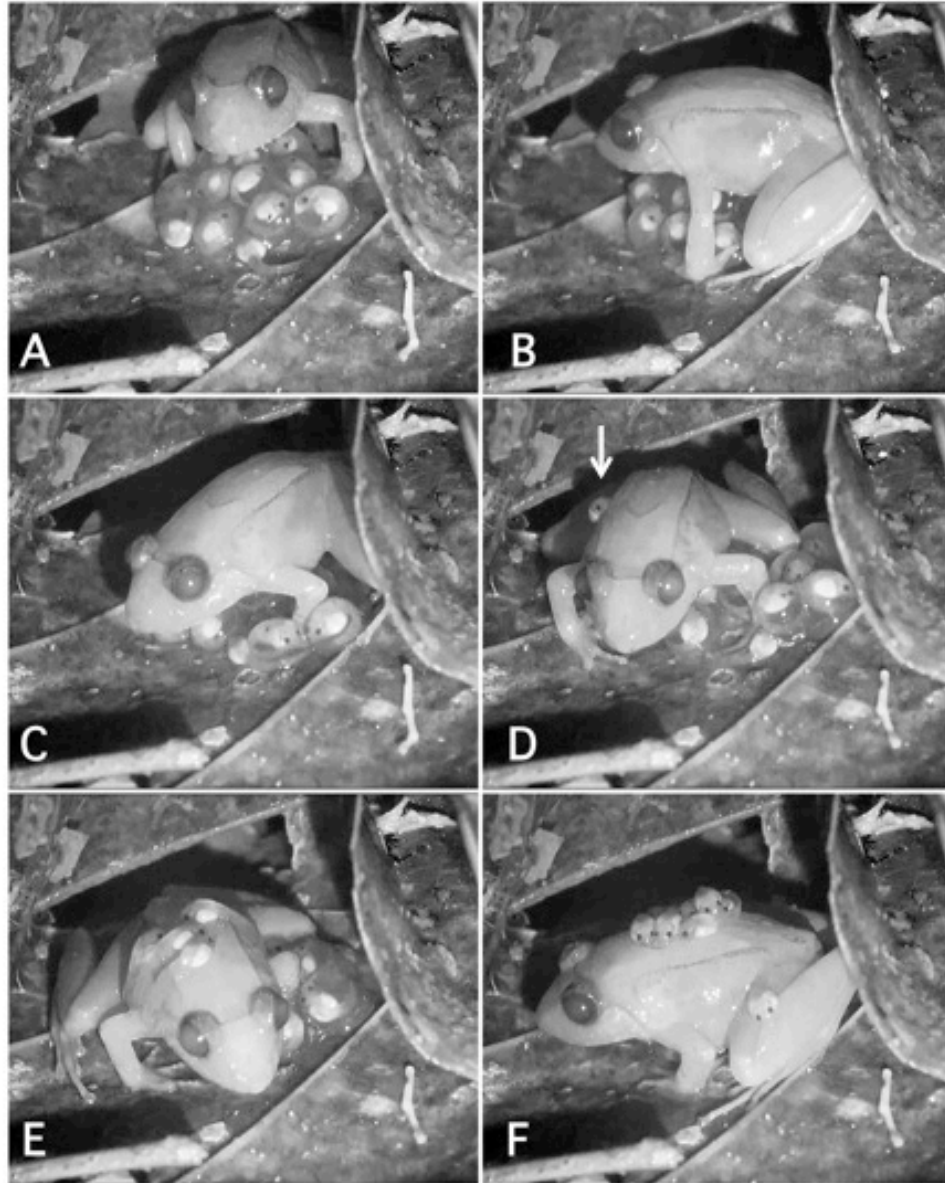


Figure 2.4. Climbing behavior of the tadpoles of *L. palavanensis* from infrared video camera footage in complete darkness. **A.** The male stimulates hatching by touching the eggs with its chin and fingers. **B.** The male positions himself on top of the eggs and twitches his abdomen and fingers. **C.** He steps on the eggs breaking the egg capsule and separating them. **D.** The first tadpole starts climbing (indicated by the arrow). The male sits on top of the now free tadpoles and waits for them to climb. **E.** The male starts to turn around while the tadpoles wriggle onto his back. After every turn he remains still for a few seconds while the tadpoles climb. **F.** The tadpoles move around and rearrange while the last tadpole makes it to the top.





Figure 2.5. Male of *Limnonectes palavanensis* with tadpoles on his back. Photos by Johana Goyes Vallejos.



Figure 2.6. Male of *Limnonectes palavanensis* depositing tadpoles in a pool formed in animal wallow.

## CHAPTER 3. TADPOLE DEPOSITION BEHAVIOR IN THE SMOOTH GUARDIAN FROG

### *LIMNONECTES PALAVANENSIS*, FROM BORNEO

#### Introduction

In species with parental care, the offspring benefit from parental decisions that increase survival (Clutton-Brock 1991). Some of these decisions include protecting the offspring from predators, competitors, desiccation, and disease, and providing resources such as food and suitable nesting sites (Refsnider and Janzen 2010). In anuran amphibians, most species that lay eggs in water, arboreal or in terrestrial environments do not provide care. However, among anuran species there is a wide variety of parental care modes, including egg guarding, egg transport, larval and froglet transport, and feeding of larvae (Crump 1995, Wells 2007). In the tropics, some frogs deposit eggs or tadpoles in small bodies of water, presumably to avoid larger aquatic environments filled with predators such as fishes.

Parent frogs may place eggs or tadpoles in water-holding leaf axils of plants such as bromeliads or in folds of fallen leaves, pitcher plants, and tree hollows (collectively called ‘phytothelmata’), as well as terrestrial sites such as small forest floor rain pools and standing bodies of water near small streams (Wells 2007). For example, the golden rocket frog *Anomaloglossus beebei* (Family: Aromobatidae) is a bromeliad specialist, with females depositing eggs in leaf axils and occasionally feeding tadpoles with unfertilized eggs (Bourne et al. 2001). The Taiwan rhacophorid frog *Kurixalus eiffingeri* lays eggs in bamboo stumps where tadpoles can finish their development in the absence of predators (Lin and Kam 2008). Some species deposit eggs in very particular habitats, such as Brazil nut capsules used by the poison dart frogs *Adelphobates castaneoticus* and *A. quinquevittatus*, and the Amazonian toad *Bufo*

*castaneoticus* (Caldwell 1993, Caldwell and de Araujo 2004), or empty snail shells used by the Guinea river frog *Phrynobatrachus guineensis* (Rudolf and Rödel 2005).

Most dendrobatid frogs lay their eggs in leaf litter and transport the tadpoles on their backs to small pools of water. These tadpole deposition sites are variable among species and include quiet streams, pools at the edges of streams, temporary ponds on the forest floor, holes in fallen logs, tree buttresses, fallen palm leaves, and bromeliad tanks. In *Oophaga histrionica*, *O. pumilio*, *O. lehmanni* and *Ranitomeya imitator*, tadpoles are transported one at a time to bromeliads and leaf axils (Silverstone 1973, Weygoldt 1980, Zimmermann 1981, Brown et al. 2010). In contrast, species such as *Colostethus panamansis*, *Allobates femoralis*, *Mannophryne trinitatis*, and *Epidobates trivittatus* carry the entire clutch at once, depositing tadpoles in relatively larger pools such as tree holes or quiet streams (Wells 1980, Höld 1983, Ringler et al. 2013). In these cases, the carrying parent may distribute the tadpoles in different pools or deposit all of them simultaneously.

While these small bodies of water usually are free of large invertebrate and vertebrate predators, they present risks of their own due to fluctuations in water volume, oxygen levels, food resources, and temperature. Also, competition and predation from conspecific and heterospecific tadpoles and some small invertebrates affect the suitability of these sites (Brown et al. 2008, McKeon and Summers 2013). Furthermore, permanency of these bodies of water oscillates with temperature and amount of rainfall, rendering them ephemeral and therefore not always available. In the face of these challenges, parent frogs are expected to choose deposition sites that minimize risks and at the same time maximize offspring survival.

Experimental studies have shown that adult frogs of some species avoid depositing eggs in pools with conspecific tadpoles that readily cannibalize eggs or newly hatched tadpoles

(Spieler and Linsenmair 1997, Summers 1999). In some species of dendrobatids, males preferentially deposit their tadpoles in large pools with abundant food resources (Jowers and Downie 2005, Poelman et al. 2013, von May et al. 2009). Both *Kurixalus eiffingeri* and *Phrynobatrachus guineensis* deposit their eggs in sites with high water-holding capacity and permanence to lessen the risk of desiccation (Diesel et al. 1995, Yi-Shian et al. 2008). However, sites with enough resources and with low desiccation and predation risks are not always available and may be dispersed in the environment, making them difficult to find. Thus, when suitable oviposition or tadpole deposition sites are scarce, parent frogs may be forced to use pools that contain predators or competitors (Murphy 2003, Rojas 2014).

We studied tadpole deposition behavior of the smooth guardian frog, *Limnonectes palavanensis* (Dicroglossidae), a small nocturnal leaf litter frog found in the primary forests of Borneo. Mated pairs lay a clutch of up to 21 eggs on land (Chapter 1). The male subsequently attends the eggs and transports the tadpoles on his back to water, where the tadpoles complete their development (Inger et al. 1986, Inger and Voris 1988, Chapter 1; Fig 3.1). Inger and Voris (1988) found *L. palavanensis* tadpoles in pools of small intermittent streams and in puddles formed after rains, including a pool in a tree hole and a pool on a fallen log. These observations suggest that tadpole deposition sites are scarce and that males might have to spend a long time looking for suitable sites.

To evaluate the occurrence of suitable tadpole deposition sites and how males choose deposition sites, we studied a population of *L. palavanensis* in Brunei Darussalam. Our main objectives were to (1) characterize potential tadpole deposition sites in our study area, (2) determine if habitat variables such as canopy cover, slope, distance to a stream, and amount of leaf litter affect the decisions of males depositing tadpoles in artificial pools, (3) test if males

prefer to deposit their tadpoles in sites without predators or potential competitors, and (4) determine if cannibalism occurs in tadpoles of this species.

## **Methods**

### *Characterization of natural tadpole deposition sites*

This study took place from August through December 2014 in the Ulu Temburong National Park, Temburong District of Brunei Darussalam. Ulu Temburong is the only national park in the country. It comprises fifty thousand hectares of lowland mixed dipterocarp rainforest. The Institute of Biodiversity and Environmental Research (IBER) manages the Kuala Belalong Field Studies Centre (KBFSC), a research facility at the heart of the Ulu Temburog National Park. The KBFSC is located at 115° 09' E, 4° 33' N and 50 – 200 m above sea level. The yearly rainfall at the site varies between 4900 and 6800 mm, with no specific dry season. However, the period with lowest amount of rainfall correspond to the months of June to August (KBFSC Weather Data 2005 – 2014). The characterization of natural deposition sites was done throughout the 2014 field season (5 months). We walked through the designated study area (ca. 1.3 ha), identifying possible natural tadpole deposition sites. We also sampled along 300 m of a small stream adjacent to our study area, looking for small pools at the edge of the stream and holes in rocks. We checked for the presence of *L. palavanensis* tadpoles, counted the number of tadpoles when present, and noted the presence of eggs or tadpoles of other species.

### *Artificial tadpole deposition sites*

In November 2013 we placed 24 artificial pools ('buckets' hereafter) of approximately 2.5 L (24 cm diameter, 10 cm depth) in transects perpendicular to the biggest stream in the



forest. Twenty buckets were placed along four different transects. The first bucket of each transect was placed 15 m from the stream, the second 30 m from the stream, the third 45 m from the stream, the fourth 60 m from the stream, and the fifth 90 m from the stream, with each transect separated by 15 m from the next one. Four additional buckets were placed haphazardly within the study area. The buckets were buried in the ground up to their rims and filled with stream water. Males of *L. palavanensis* were not observed using the buckets during the 2013 field season. The buckets were left in place in the forest for ten months. In August 2014, we started the surveys for *L. palavanensis* tadpoles by checking each bucket every other day. We recorded the presence or absence of tadpoles, and, if present, counted the number of tadpoles. When found, the tadpoles were removed from the buckets and taken to the laboratory in the field station for measurements. The tadpoles were kept in tanks with leaf litter until the end of the survey when they were released in natural pools. Tadpoles and eggs of other species were removed as well.

For each of the buckets, we recorded depth of leaf litter surrounding the bucket, canopy cover, and slope. To determine the amount of leaf litter around a bucket, we took four measurements around the bucket using a thin metallic rod to pierce through the leaves until it reached the soil. The four measurements were then averaged. We used a 60D EOS Canon camera with an 18 mm lens to estimate canopy cover. The photographs were taken at ground level, with the camera centered on the bucket and later analyzed using the software ImageJ. To determine the slope where a given bucket was located, we used ©Bushnell Laser Rangefinder (Elite 1600 ARC). This survey was carried out from August 18, 2014 to October 5, 2014.

To determine if any of the variables measured (i.e. leaf litter depth, canopy cover, slope and distance to the stream) had an effect on the presence or absence of tadpoles in the buckets,

we fitted generalized linear mixed effect models using the package lme4 (Bates et al. 2015) in R (R Development Core Team 2015). We modeled linear regressions ( $y = \beta_0 + \beta_1\chi$ ) with a binomial distribution using a logit link function for each of the parameters and using ‘bucket’ as a random variable for all the linear models to account for repeated measures through time. In addition, we included a global model with all the covariates as fixed effects and a null model (no covariates). We used the Akaike’s Information Criterion (AIC) to rank the set of six models and the values of  $\Delta AIC < 2$  ( $\Delta AIC_i = AIC_i - AIC_{min}$ ) to find the best models.

#### *Tadpole deposition site choice experiments*

We conducted two experiments using pairs of buckets to test if the presence of predators or conspecifics affects whether or not a male deposits tadpoles. For both experiments we set up ten pairs of buckets of the same size used in the tadpole surveys. The paired buckets were 25 to 30 cm apart. The paired buckets were placed haphazardly throughout the study area, and each pair was at least 15 m from the next nearest pair. We covered the bottoms of the buckets with a fine layer of leaf litter and filled them with stream water. For experiment 1, to test if the presence of conspecifics tadpoles affects tadpole deposition by males, we deposited two tadpoles of *L. palavanensis* in one bucket, while the other remained empty. For experiment 2, to test if the presence of a predator affects tadpole deposition behavior on males of *L. palavanensis*, one of the buckets contained a dragonfly larva (Order Odonata: Suborder Anisoptera) enclosed in a mesh bag, and the other bucket contained an empty mesh bag. The mesh bag allowed the dragonfly larva to swim freely in the bucket, but prevented it from eating tadpoles.

For both experiments, the paired buckets were checked every other day for the presence or absence of tadpoles, and for the number of tadpoles. If present, the newly deposited tadpoles



were removed and brought to the lab and kept until the end of the experiment so the buckets were available for other males to deposit their tadpoles under the same experimental conditions. The position of the buckets with predator/conspecifics was switched each time after the buckets were checked. To test if males preferred to deposit their tadpoles in buckets free of conspecific tadpoles or a predator, we used a McNemar's Chi-squared test with continuity correction used for paired binary response data with “no preference” as our null hypothesis. The McNemar's tests for both predator and presence of conspecifics were done in R (R Development Core Team 2015). The conspecific tadpole experiment took place between October 6, 2014 and November 9, 2014, and the predator experiment between November 11, 2014 and December 13, 2014.

#### *Cannibalism experiment*

From November 13, 2014 to December 13, 2014 we formed 18 pairs of tadpoles to test if cannibalism occurs in tadpoles of *L. palavanensis*. The pairs were formed randomly using tadpoles found in the buckets from the tadpole site choice experiments and tadpoles found in natural pools more than 500 m away to ensure the two tadpoles were not related. We placed a small tadpole with a large one and photographed them to subsequently measure the differences in size using the software ImageJ. Each pair of tadpoles was placed in a small plastic container (16x11x6 cm) on a dark background with a mesh lid. Each container was filled with 2 grams of crushed leaf litter and 200 ml of water. We checked the pairs three times per day for four weeks to see if cannibalism had occurred.

## Results

### *Characterization of tadpole deposition sites*

We found only 10 natural pools throughout our study area that appeared to be suitable for tadpole deposition. These pools were surveyed regularly for five months for presence of *L. palavanensis* tadpoles. We found tadpoles only three times: in a pool of an intermittent stream (N = 19 tadpoles; Fig 3.2A), in a pig wallow (N = 35 tadpoles), and in a water-filled depression on a boulder next to the stream (N= 30 tadpoles; Fig 3.2B). Eggs and larvae of a microhylid frog, *Chaperina fusca*, were found in two of the remaining seven pools, but we never found *L. palavanensis* tadpoles or eggs and tadpoles of other species in those pools.

### *Artificial tadpole deposition sites*

Of the 24 buckets surveyed in 2014, males of *L. palavanensis* used 18 of them at least once to deposit tadpoles. We recorded 56 deposition events with the number of tadpoles per deposition ranging from 1 to 17 tadpoles (median number of tadpoles = 4; Fig 3.3). Twenty buckets were used at night by *Chaperina fusca*, *Kalophrynus pleurostigma* and *Microhyla borneensis* (Microhylidae) as calling sites and breeding pools. Sometimes, up to 10 adults of *C. fusca* were found in one bucket at a time, and up to two calling males of *K. pleurostigma*. The adults, tadpoles and eggs of these species were relocated after every survey. Small dragonfly larvae (< 1cm) were found and removed from the buckets on seven occasions.

A summary of the values of each environmental variable measured and all the  $\Delta AIC$  values comparing the six different models on the effect of leaf litter, slope, canopy cover and distance to the stream on the probability of a male *L. palavanensis* depositing his tadpoles in an artificial pools are presented in Table 3.1 and Table 3.2 respectively. The model that included

slope as a covariate was best supported by the data (Table 3.2). The steeper the slope on which the artificial pool was located, the less likely a male was to deposit tadpoles in those buckets (Fig 3.4). The linear regression of the probability of finding tadpoles, as a function of the variable “slope” was significant, indicating that slope is negatively correlated with the probability of a male depositing tadpoles in a given bucket ( $\beta_1 = -0.51$ , 95%CI =  $-0.94 - -0.08$ ,  $P=0.021$ ). None of other variables (i.e. leaf litter, canopy cover and distance to the stream) had a significant effect on the probability of finding tadpoles (all  $P > 0.05$ ).

#### *Tadpole deposition site choice experiments*

Males of *L. palavanensis* used seven of the ten pairs of experimental buckets to deposit tadpoles. We observed males carrying tadpoles at night (2030h – 0000h) and depositing them in the buckets on five occasions (Fig 3.5). We estimated the number of tadpoles carried by each male and compared it with the number of tadpoles found in the buckets the next morning (number of tadpoles = 14, 15, 12, 14, and 11). In all five cases, our estimates of the number of tadpoles on the backs of the males matched the number of tadpoles found the following day. In addition, all of the observed males split the tadpoles between the two buckets.

In Experiment 1, testing the effect of conspecific tadpoles, we recorded 21 tadpole deposition events with a maximum of five repeated deposition events per pair of buckets throughout the experiment (range = 2 – 5; Table 3.3). Consecutive tadpole deposition events in the same pair of buckets occurred within a maximum of five days. On average, it takes ten days for a clutch of eggs to hatch; therefore, it is unlikely that the same male returned to the same pair of buckets to deposit his tadpoles. Males did not exhibit any preference between the empty bucket and the bucket occupied by conspecific tadpoles (McNemar's chi-squared = 0.125,  $df = 1$ ,

$P = 0.7237$ ; Fig 3.6). We found tadpoles in both buckets on 13 occasions, with a maximum combined number of tadpoles of 21 (median = 12, range = 3–21). We tested for differences in total length between the tadpoles found in the empty bucket and the bucket with the conspecific tadpoles (when number of tadpoles  $> 2$  in both buckets), and in all but one case there were not significant differences in size (all  $P > 0.1$ ). In the exceptional case, 27 tadpoles were deposited, 7 tadpoles in the occupied bucket and 20 tadpoles in the empty bucket. After inspection of the development of the ventral intestinal coil and size, we sorted the tadpoles into three groups that differed in developmental stage; these included 7, 8, and 12 tadpoles each, and found significant differences in size among these groups ( $P < 0.05$ ), suggesting that three different males deposited the tadpoles in the two buckets over a span of two days.

In Experiment 2, testing the effect of a predator on tadpole deposition behavior, we recorded 12 deposition events with a maximum of three repeated deposition events per pair of buckets throughout the experiment (range = 1–3; Table 3.4). Males did not show a preference for depositing tadpoles in buckets without a predator (McNemar's chi-squared = 2.28,  $df = 1$ ,  $P = 0.1306$ ; Fig 3.6), and deposited at least 3 tadpoles in the bucket with the predator in 50% of the deposition events. On five occasions, we found tadpoles in both buckets, with a combined maximum number of tadpoles of 14 (median = 11, range = 6–14). The largest clutch size observed in this species is 21 eggs, so we assumed that the tadpoles found in both buckets belonged to the same clutch. To confirm this, we compared the developmental stage and total length of the tadpoles, and in all but one case, the sizes of the two groups were the same (all  $P > 0.1$ ).

The mean  $\pm$ SD number of tadpoles found per deposition event was  $11 \pm 5$  (range = 2–21) in the presence of conspecific tadpoles experiment, and  $9 \pm 5$  (range = 1–15) in the predator

experiment. Adults and eggs of *C. fusca*, *K. pleurostigma* and *M. borneensis* constitute 63.5% of the total number of sampling events in the presence of conspecifics experiment (present in both buckets) and 42.3% in the predator experiment. Adults and eggs of these microhylid species sometimes co-occurred with tadpoles of *L. palavanensis*.

#### *Cannibalism experiment*

The total length of the small tadpoles ranged from 14.6 mm to 20 mm (mean  $\pm$ SD = 17.3  $\pm$ 1.7 mm), while the total length of the big tadpoles ranged from 16.8 mm to 23.6 mm (mean  $\pm$ SD = 21.3  $\pm$ 2.0 mm) The paired tadpoles differed in total length by 0.73–7.69 mm and by 0.13–1.50 mm in width. Cannibalism did not occur in any of the pairs of tadpoles.

#### **Discussion**

Low availability of tadpole deposition sites seems to be the main factor affecting tadpole deposition choice in *L. palavanensis*. We found that tadpole deposition sites are scarce throughout the forest and it seems that not all water pools are suitable for tadpole deposition. In *Allobates femoralis*, a Neotropical diurnal leaf litter frog with similar parental behavior, natural pools are also scarce and ephemeral. These sites were similar to those found in this study (a rain pool on the forest floor, a palm frond, a flooded animal burrow, and a pool on a fallen log). Thus, in this species, adults rely heavily on artificial pools for tadpole deposition (Pasukonis et al. 2016). There are several characteristics that can influence the suitability of offspring deposition sites, including the permanence and duration of the water habitat. In some species of frogs where adults deposit their eggs in small bodies of water, ovipositing pairs show a preference for sites that can hold water for several weeks (Rudolf and Rödel 2005). In our study area, the type of soil

in the forest floor does not allow for rain pools to last very long, rendering potential tadpole deposition sites ephemeral. Of three natural pools observed to have *L. palavanensis* tadpoles, the common feature is the durability of the pool. The rocky depression near to the stream seems to be maintained by frequent rainfall, while the pool in the intermittent stream is sustained by continuous dripping from a bigger stream. Also, the rocky bottom of this pool prevents, to some extent, water filtering through the soil. The animal wallow was the only natural pool used by *L. palavanensis* that showed substantial fluctuations in the amount of water present at a given time. During heavy rains, the wallow was a big pool holding water for about a week, but without rain to fill the pool, the wallow became an aggregation of several smaller pools containing between 2–16 tadpoles of *L. palavanensis* (Goyes J. pers. obs).

Males of *L. palavanensis* have been observed carrying 8 to 15 tadpoles (median = 13) on their backs (Chapter 2), and during this study, five males were observed depositing tadpoles in the artificial buckets (range = 11–15, median = 14). The larger numbers of *L. palavanensis* tadpoles found in the natural pools (19–35 tadpoles) suggest that more than one male deposited tadpoles in the pools, providing further evidence of limited tadpole deposition sites.

In our survey of artificial pools, the probability of a male depositing tadpoles in one of the artificial pools did not depend on the distance to the stream, the amount of leaf litter around the pool, or the amount of canopy cover. However, the slope at the location of the artificial pool did influence tadpole deposition. Males and females of *L. palavanensis* can be found calling in very steep areas and the artificial pools were located in areas where calling males had previously been heard. However, males may avoid pools in steep areas because the inclination of the forest floor does not allow natural pools to hold water, although this would not be true for the buckets.

In addition, heavy rains could easily wash out the tadpoles in pools found on steep slopes (Brown and Iskandar 2000).

Artificial pools were readily used by three other species of frogs as oviposition sites even before our first observations of tadpole deposition by males of *L. palavanensis*. The presence of eggs and larvae of these species did not seem to affect tadpole deposition by *L. palavanensis*, because tadpoles of this species co-occurred with eggs and tadpoles of the other species. This rapid colonization of artificial pools by other species could also indicate scarcity of breeding sites for all species that use small pools. It is unknown how these species, including *L. palavanensis*, find these pools. Males of *L. palavanensis* do not call while carrying the tadpoles, but they might use heterospecific acoustic cues to find pools. Some studies in temperate frogs that breed in ephemeral pools suggest that calls from the first frogs to colonize could aid in finding pools of water for reproduction (Buxton et al. 2015). Regardless of how they locate these artificial pools, males of *L. palavanensis* probably learn their location over time and return repeatedly to deposit their offspring, as observed in species with tadpole transport behavior in the New World (Pasukonis et al. 2016).

During the surveys of the artificial pools, we observed a wide range in the number of tadpoles deposited in each bucket (1–17, median = 4). In the poison frog *Allobates femoralis* larval survivorship is high, and males sometimes carry entire clutches (up to 20 tadpoles) and distribute their offspring in different pools when available, but sometimes travel long distances if suitable sites are rare (Ringler et al. 2013). In *L. palavanensis*, a few cases of clutches with low survival rates have been observed in the lab in the absence of terrestrial predators (Chapter 2), but most clutches had a survival rate of 75% or higher. Thus, we cannot explain the low number

of tadpoles per deposition in *L. palavanensis* without further studies in offspring survival and larval transport logistics.

In species with parental care, offspring benefit from parental decisions that provide reasonable protection against predators, competitors and desiccation (Wells 2007). In the Peruvian poison frogs *D. imitator* and *D. variabilis*, males avoid depositing tadpoles in pools with predators and heterospecific competitors (Brown et al. 2008). Males of *D. ventrimaculatus* avoid depositing tadpoles in pools with large conspecific tadpoles to avoid cannibalism of their offspring (Summers 1999, Summers and Symula 2001). Males of *L. palavanensis* did not avoid pools with conspecific tadpoles and often split their clutches between the two available buckets. In this study, males of *L. palavanensis* divided their clutch between two nearby buckets. So, whether or not males split their clutches may depend on pool availability and awareness of the location of neighboring pools. Nonetheless, this clutch partitioning behavior is advantageous if males maximize their fitness by depositing small numbers of tadpoles in different pools since this could minimize the risks associated with small pools (Erich et al. 2015).

Conspecific tadpoles do not present a threat for the newly deposit tadpoles, since there is no cannibalism in this species. In addition, tadpoles feed from detritus on the bottom of the buckets and it is unlikely that food resources are limited. In some species high tadpole densities in small pools trigger cannibalistic behavior (Caldwell and de Araujo 1998). This does not seem to be the case in *L. palavanensis* where a large number of tadpoles can be found in one pool. In contrast to some dendrobatids, males of *L. palavanensis* may not be capable of detecting the presence of resident tadpoles by movement alone, as the tadpoles are very inactive, resting among the leaves on the bottom of the pool. As a result, males may not be able to discriminate between the experimental buckets. However, in the Peruvian poison frog *Ranitomeya variabilis*



it has been demonstrated that adults can discriminate between pools with cannibalistic and non-cannibalistic tadpoles based on chemical cues, depositing more tadpoles in pools with non-cannibalistic tadpoles (Schulte et al. 2011). Whether or not males of *L. palavanensis* can detect the presence of conspecific tadpoles by means of chemical cues is unknown.

The presence of conspecifics also might be an indicator of availability of food resources and water permanence (Poelman and Dicke 2007). Water permanence is critical depending on the time needed to reach metamorphosis. We do not have information in the field about how long it takes for the tadpoles to metamorphose. However, the tadpoles used as “residents” for this experiment were about a week old, based on size. After approximately three weeks, all the residents had developed hind legs (Gosner stage 39 – 40) and by the end of the experiment (~ 1 month) their bodies elongated and the forelimbs started to emerge (Goyes J. pers. obs.). This indicates that, natural pools must last at least 30 days for successful development of the larvae.

Males did not discriminate between the buckets where a predator was present or absent. Males are expected to make choices that maximize offspring survival; therefore it is surprising that males of *L. palavanensis* sometimes deposit some, and in other cases all, of their tadpoles in the bucket with a predator. It is possible that males are unable to effectively detect odonate predator chemical cues in the water and therefore they do not differentiate the predator bucket from the predator-free bucket. An alternative explanation is, that predation risk is low even in the presence of a predator, because tadpoles of *L. palavanensis* rely heavily on camouflage and inactivity for protection from predators, feeding on the bottom of the pool under the leaf litter without swimming actively. Hence, predation is minimized, because many species of odonate larvae rely on movement for prey capture (Chovanec 1992).

Our results suggest that factors other than possible competitors or predators affect the decision to deposit tadpoles in a pool. Low density of deposition sites and water permanency may be the main drivers in the tadpole deposition behavior of males of *L. palavanensis*. The few available sites in the field need to be permanent enough to allow the development of the tadpoles. Males then should deposit all their tadpoles in one pool when the probability of finding another pool is low or requires traveling long distances, which in turn increases predation risk, dehydration of the tadpoles and depletion of their yolk reserves. However, given the opportunity, males divide the number of tadpoles between two nearby pools increasing the probabilities of survivorship in case of desiccation of deposition sites. Other aspects such as pool size and amount of food resources need to be studied in order to fully understand the deposition behavior of *L. palavanensis*.

Availability of natural pools may influence the mating system of *L. palavanensis*. Shortage of deposition sites is expected to increase the number of days a male *L. palavanensis* carries his tadpoles before a suitable site is found. During this time, males cannot acquire additional mates, which in turn will affect the operational sex ratio (OSR) of the population. The operational sex ratio (OSR) determines the number of females and males available for mating at a given reproductive event. Whether the OSR is biased towards one sex or the other depends on how long it takes for an individual to get back into the mating pool, defined as the “time-out.” In *L. palavanensis* egg attending takes an average ten days (Chapter 2). Additional days trying to find deposition pools after the clutch has hatched will extend the time-out of the males and females will suffer from reduced mating opportunities. This could lead to reversal of the usual sex roles in mate-searching or courtship, with females calling to signal their presence to males and perhaps competing among themselves for access to individual males.

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Figure 3.1. Male of the smooth guardian frog *Limnonectes palavanensis* carrying four tadpoles on his back. Males sometimes transport up to 17 tadpoles at a time to small bodies of water.

Photo by Johana Goyes Vallejos.



Figure 3.2. Natural tadpole deposition sites of *Limnonectes palavanensis* at the Ulu Temburong National Park study site in Brunei Darussalam. A. Pool of an intermittent stream, B. Water-filled depression on a boulder next to a stream.



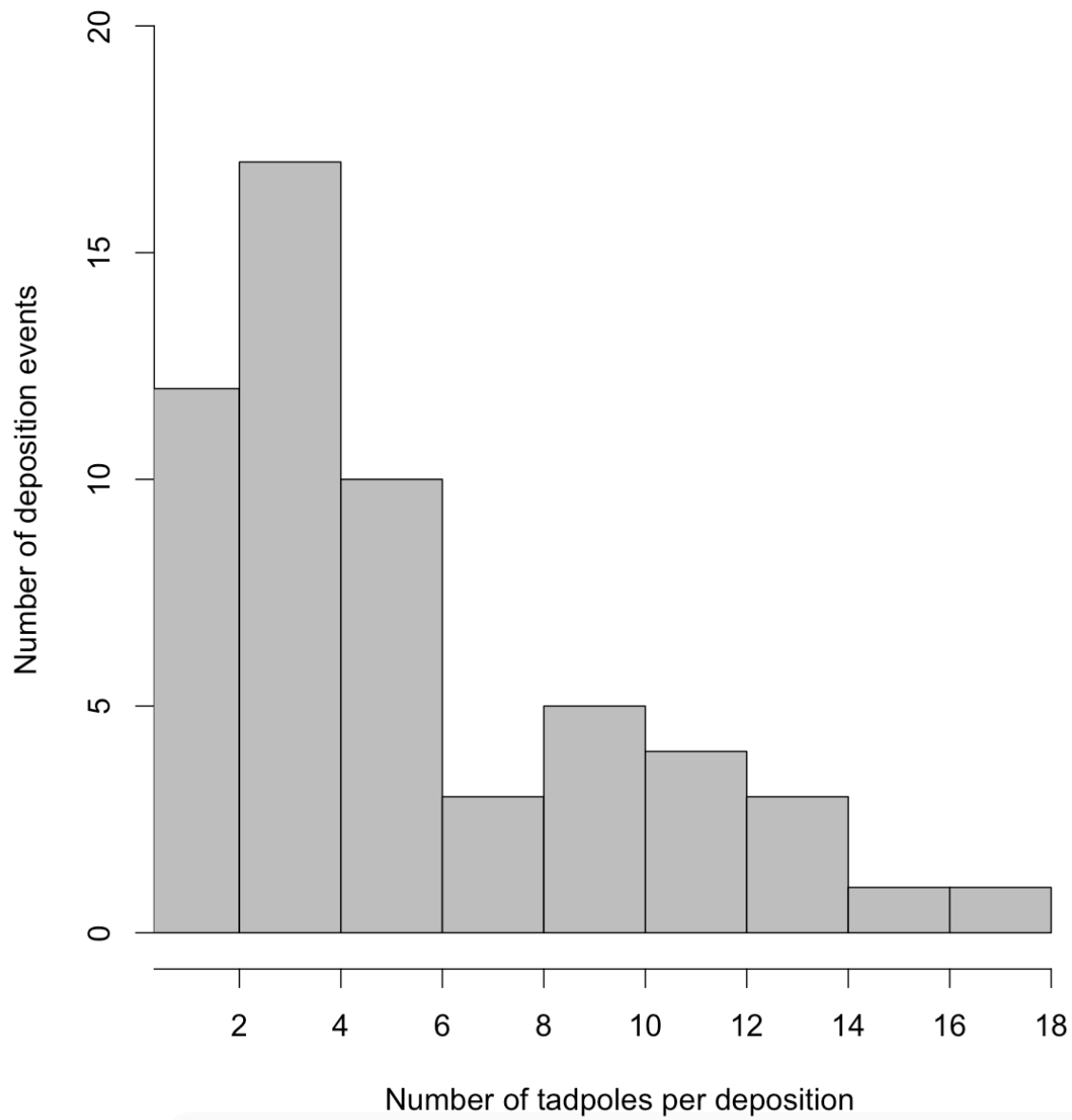


Figure 3.3. Number of *Limnonectes palavanensis* tadpoles per deposition event found in the artificial deposition sites. Median number of tadpoles = 4 (range = 1–17).

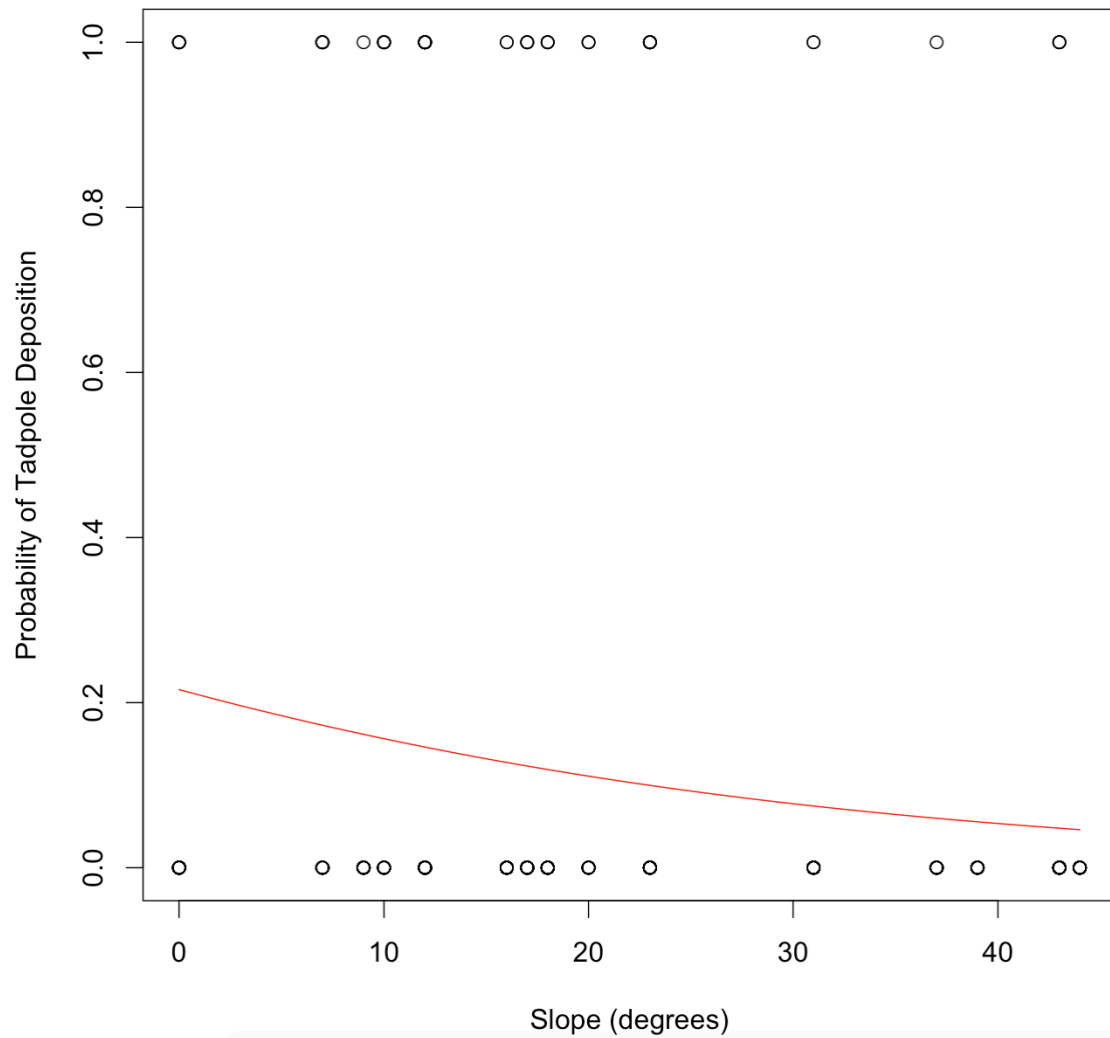


Figure 3.4. Effect of the slope on the probability of a male depositing tadpoles in an artificial pool. The circles indicate data obtained in the field and the line shows the predicted probabilities using a binomial logistic regression.



Figure 3.5. Male *Limnonectes palavanensis* carrying tadpoles at the edge of an artificial pool. The male later entered the pool and deposited his tadpoles. Photo by Johana Goyes Vallejos.

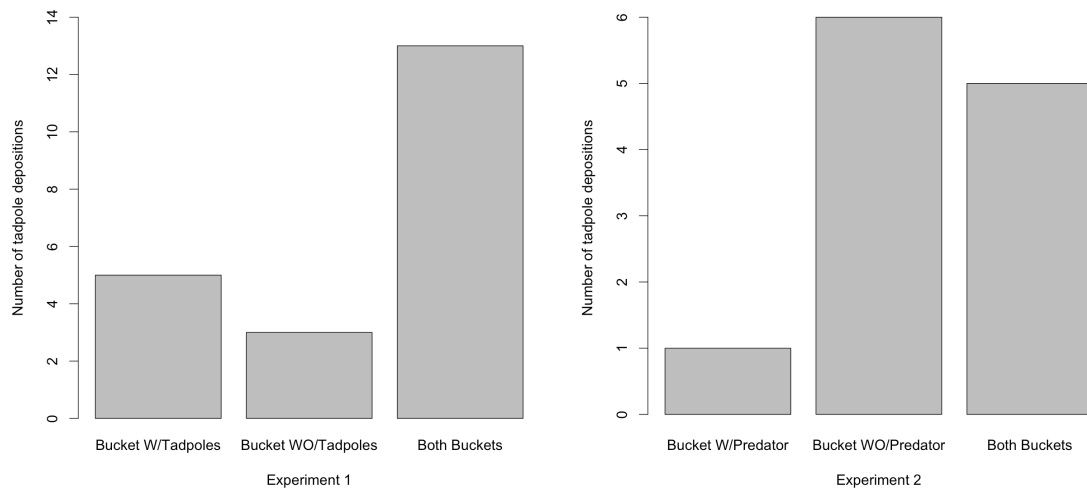


Figure 3.6. Number of tadpole deposition events of males of *L. palavanensis* in Experiment 1 (presence of conspecifics) and Experiment 2 (presence of a predator). Males do not avoid depositing their tadpoles in pools containing conspecifics or predators (McNemar's test,  $P > 0.1$ ).

Table 3.1. Summary of the mean  $\pm$ SD leaf litter depth, slope and canopy cover measured for each of the 24 artificial pools established in the study area.

Variable	mean $\pm$ SD	Min–Max
Leaf litter depth (cm)	2.7 $\pm$ 1.1	1.6–5.5
Slope (°)	20.8 $\pm$ 13.1	0–44
Canopy cover (%)	87.0 $\pm$ 7.5	64.5–97

Table 3.2. Summary of the model comparison and Akaike's Information Criteria values (AIC) and  $\Delta$ AIC values comparing different models on the effect of leaf litter, slope, canopy cover and distance to the stream on the probability of tadpole deposition of *L. palavanensis* in artificial pools.

<b>Models</b>	<b><i>k</i></b>	<b>AIC</b>	<b><math>\Delta</math>AIC</b>	<b><math>w_i</math></b>
Model (Tadpoles ~ slope + (1 Bucket))	3	324.0	0	0.55
Model (Tadpoles ~ Distance + (1 Bucket))	3	326.4	2.40	0.17
Model (Tadpoles ~ 1 + (1 Bucket))	2	327.5	3.54	0.09
Model (Tadpoles ~ litter + slope + canopy + distance + (1 Bucket))	6	327.7	3.74	0.09
Model (Tadpoles ~ Canopy + (1 Bucket))	3	328.6	4.60	0.05
Model (Tadpoles ~ litter + (1 Bucket))	3	329.1	5.10	0.04

Table 3.3. Tadpole deposition data for the experiment using conspecific tadpoles. The numbers indicate the total of tadpoles per deposition event in each bucket. Tadpole total length at deposition (TTL) in mm, measured from the snout to the tip of the tail. Bucket ID indicates the transect number (MT) and the distance to the stream in meters.

Pair	Bucket ID	With Tadpoles	W/O Tadpoles	Total	mean±SD TTL (mm)
1	MT1 60	3	2	5	12.0±1.2 (11.2–14.2)
1	MT1 60	13	1	14	13.8±0.9 (11.6–14.9)
2	MT1 45	7	6	13	13.4±0.9 (11.9–14.9)
2	MT1 45	4	11	15	13.9±0.4 (13.2–14.8)
3	MT2 90	12	0	12	12.3±0.9 (10.7–14.0)
3	MT2 90	0	11	11	14.4±0.6 (13.3–15.4)
6	MT3 45	8	0	8	13.9±0.6 (12.7–14.5)
6	MT3 45	0	2	2	13.6±0.1 (13.6–13.7)
6	MT3 45	1	2	3	13.2±0.2 (12.9–13.5)
6	MT3 45	6	6	12	12.7±0.4 (11.9–13.3)
7	MT3 30	7	0	7	12.9±0.7 (11.4–13.7)
7	MT3 30	7	0	7	13.8±0.4 (13.1–14.3)
9	MT4 60	7	1	8	15.4±0.5 (15.0–16.0)
9	MT4 60	2	19	21	14.2±0.9 (12.3–15.8)
9	MT4 60	7	10	17	13.4±0.4 (12.0–14.1)
9	MT4 60	5	6	11	14.5±0.8 (12.6–15.3)
10	MT4 45	17	0	17	13.3±0.4 (12.2–14.2)
10	MT4 45	6	4	10	15.1± 0.3(14.4–15.7)
10	MT4 45	3	2	5	14.6±0.4 (14.1–15.1)
10	MT4 45	0	12	12	13.9±0.6 (12.5–14.8)
10	MT4 45	7	8	15	14.7±0.5 (14.0–15.3, n=7)* 15.7±0.6 (14.6–16.3, n=8)*

\* Significant differences ( $P<0.05$ ) between the tadpoles deposited in the bucket with tadpoles (n=7) and the bucket without the tadpoles (n=8)

Table 3.4. Tadpole deposition data for the predator experiment. The numbers indicate the total of tadpoles per deposition event in each bucket. Tadpole total length at deposition (TTL) in mm, measured from the snout to the tip of the tail. Bucket ID indicates the transect number (MT) and the distance to the stream in meters.

<b>Pair</b>	<b>Bucket ID</b>	<b>With Predator</b>	<b>W/O Predator</b>	<b>Total</b>	<b>mean±SD TL (mm)</b>
1	MT1 60	4	2	6	14.1±0.5 (13.4–14.7)
2	MT1 45	6	5	11	13.6±0.9 (12.1–14.7)
2	MT1 45	3	4	7	12.0±0.6 (11.2–12.7)
3	MT2 90	6	6	12	13.7±0.5 (12.6–14.4)
3	MT2 90	0	1	1	15.9
3	MT2 90	12	0	12	14.5±0.4 (13.6–15.2)
6	MT3 45	9	5	14	14.0±1.0 (12.2–15.6)
6	MT3 45	0	14	14	14.2±0.4 (13.4–15.0)
7	MT3 30	0	15	15	12.8±0.8 (11.0–14.1)
7	MT3 30	0	4	4	13.7±0.1 (13.5–13.8)
9	MT4 60	0	7	7	13.9±0.9 (12.4–14.8)
10	MT4 45	0	6	6	13.1±1.2 (11.1–14.7)



**CHAPTER 4. CALLING BEHAVIOR OF MALES AND FEMALES OF *LIMNONECTES*  
*PALAVANENSIS*, A BORNEAN FROG WITH MALE PARENTAL CARE AND POSSIBLE SEX-  
ROLE REVERSAL**

**Introduction**

In most birds, acoustic insects, and frogs, acoustic signals are used to attract or court prospective mates (Andersson 1994, Wagner and Reiser 2000, Gerhardt and Huber 2002, Searcy and Nowicki 2005, Rebar et al. 2009). In most cases, males devote more time and effort to acoustic displays than do females. Nevertheless, there is considerable variation in signaling sex roles within and among clades. For example in passerine birds, species with male-only song are common in the temperate zone. In tropical passerines, females sometimes sing as much as males and males and females often participate in synchronized duets (Slater and Mann 2004). In some clades, singing by both males and females appears to be the ancestral condition, with female song having been lost in species that moved into the temperate zone (Price et al. 2009).

In many acoustic insects, females respond to the male advertisement signal with a courtship song, which may be similar to calls of males or different (Gerhardt and Huber 2002). In some other cases, females respond shortly after the male's call, leading to a duet that is essential for successful courtship and mating (Wells and Henry 1992, Cooley and Marshall 2001, Rodriguez et al. 2004). Examples of acoustic insects in which females call more often than males are unknown.

In most species of frogs, males produce advertisement calls to attract conspecific females or advertise ownership of territories to other males (Wells 1977). In some

species, males produce a distinct courtship call when females approach their calling sites, possibly providing cues that assist females in locating males (Gerhardt and Huber 2002, Wells 2007). Female frogs of a few species also vocalize, despite having a relatively under-developed larynx compared to that of males (see Suthers et al. 2006 for a case of reverse sexual dimorphism in larynx size). In species with female calling, the most common context is for females to respond to the male advertisement call with a courtship call (Wells 2007). Generally these calls are distinct from male calls, being shorter and of much lower amplitude. Female frogs seldom call spontaneously or in the absence of males. Often female courtship calls stimulate the males, which sometimes respond with a call distinct from the advertisement call (Given 1993, Judge et al. 2000). In some cases, the female call may serve as a signal of reproductive receptivity or reveal the female's location (Emerson 1992, Bush et al. 1996). Male vocal responses to female calls probably assist females in locating males in hidden locations or acoustically complex environments (Marquez and Verrell 1991, Tobias et al. 1998, Bosch 2001, Shen et al. 2008).

In birds, cases of females signaling more frequently than males are rare and often associated with partial or complete reversal of the usual sex roles in courtship and mating (Goymann et al. 2004, Ekstrom et al. 2007, Price et al. 2008). Such sex-role reversal is related to males assuming expensive parental care duties, which removes them from the pool of mates available to females, or to population sex ratios heavily skewed toward females. Thus, males become a limiting resource for females, and females are expected to compete for access to males (Trivers 1972, Emlen et al. 1998, Kokko and Jennions 2008). A well studied species in which the mating system is sex-role reversed is the African black coucal (*Centropus grillii*). In this species, females are larger than males and

aggressively defend territories, while males perform all of the parental care. In a given season, males can raise up to four clutches, whereas females on average produce nine clutches. Hence, the 'time out' is higher for males than females and thus females compete more intensely for mates. In addition, females are more conspicuous and their vocal activity substantially exceeds that of the males, a behavior typically associated with a sex-role reversed mating system (Andersson 1995, Illes and Yunes-Jimenez 2009, Geberzahn et al. 2009, Geberzahn et al. 2010)

Clear cases of sex-role reversal in frogs have not been reported, despite the prevalence of paternal care and the wide variety of mating systems observed in anurans. In some species of dendrobatids with male parental care, females actively court the males and sometimes engage in female-female agonistic behaviors. However, males still do most of the calling to attract mates, compete with each other for mating opportunities, and can care for more than one clutch at a time, meaning that parental care is not limiting mating opportunities for males (Wells 1980, Wells 1978, Summers 1989, Ursprung et al. 2011). One possible exception is the Majorcan midwife toad, *Alytes muletensis*. Males call from crevices and hidden locations, often widely separated from one another. Females respond to the male advertisement call with a soft courtship call. This reciprocal call aids both males and females in finding each other. Females of *A. muletensis* sometimes initiate courtship by calling spontaneously, but they only do so when they are heavily gravid and in danger of losing their eggs (Bush et al. 1996). During the breeding season, the operational sex ratio (OSR) can vary, and when the OSR is female biased, females wrestle in contests for access to males, although males also can fight for access to females (Bush 1997, Bush and Bell 1997). After amplexus and oviposition, males

intertwine the eggs around their legs and brood them until the eggs are ready to hatch. Males in this population rarely care for more than one clutch at a time, even though a female-biased sex ratio should increase mating opportunities for males (Bush 1996). Thus, males may be a limiting resource for females, leading to sex-role reversal in some aspects of courtship and mating.

We studied the smooth guardian frog (*Limnonectes palavanensis*) (Anura: Dicroglossidae) on the island of Borneo. Parental care is performed exclusively by males, which not only care for eggs, but also transport tadpoles to water (Inger et al. 1986, Inger and Voris 1988). Males give advertisement calls to attract mates, but do so very infrequently from widely spaced locations on the forest floor. Females respond to male advertisement calls with a soft short-range call, but they also call spontaneously in the absence of males. Males respond to the female call with a distinctive courtship call. With male parental care and female calling behavior, *L. palavanensis* offers a unique opportunity to investigate the possibility of a reversal of the typical anuran sex roles in calling behavior. The aims of this study were to (1) characterize the vocal repertoire of males and females of *L. palavanensis*, (2) determine the behavioral contexts in which males and females vocalize and interact with one another, and (3) quantify calling rates of males and females in the field.

### *Study Species*

*Limnonectes palavanensis* is a small leaf-litter frog found in primary and old secondary rainforests of Borneo and the Palawan Islands of the Philippines (Fig 4.1). Males call at night from the leaf litter on the forest floor, although the structure of the call

has not been published. *Limnonectes palavanensis* belongs to a genus of Southeast Asian frogs that has some unusual sexual characters. Males in some species of the genus lack vocal sacs and have been called “voiceless frogs” (Emerson 1992), although males in some species without vocal sacs produce advertisement calls (Matsui 1995, Orlov 1997). The clade also has been grouped under the name “fanged frogs,” because many species of *Limnonectes* have bony projections on the lower jaw, in addition to enlarged heads and hypertrophied jaw muscles used in male-male combat over access to females (Emerson et al. 2000). Females of *Limnonectes* typically lack fangs and are smaller than the males, a pattern opposite to that generally observed in anurans; usually females are larger than males (Wells 2007). Both males and females of *L. palavanensis* lack both vocal sacs and fangs, males do not have enlarged heads, and females are larger than males, the reverse of the pattern normally seen in this clade.

Within *Limnonectes* there is an extreme diversity in reproductive modes, from laying eggs in small streams or rock pools with no parental care (*L. kuhli*, Tsuji 2014), egg deposition in terrestrial nests with tadpoles developing in the nest (*L. limborgi*, Rowley and Altig 2012), egg brooding in muddy banks (*L. arathooni*, Brown and Iskandar 2016), to more complex modes like internal fertilization and tadpole viviparity (*L. larvaepartus*, Iskandar et al. 2014). *Limnonectes palavanensis* is the only species in the genus apart from *L. finchi* that is known to exhibit exclusive male parental care of the eggs and subsequent tadpole transportation to small pools of water, but details of its natural history have not been studied (Inger et al. 1986, Inger and Voris 1988). The taxonomy and phylogenetic relationships within *Limnonectes* have yet to be resolved, making it difficult to trace the evolution of these reproductive modes. The latest

molecular phylogenies suffer from missing data, either in species sampling, geographic region, or the number of nuclear and mitochondrial markers used in the analysis.

Although Frost (2016) recognized 65 species of *Limnonectes*, the number is likely to increase because many populations once thought to be members of a single widespread species now appear to be complexes of cryptic species, many of which are separated by geographic barriers (McLeod et al. 2012, Iskandar et al. 2014, Matsui and Nishikawa 2014, Matsui et al. 2014, Aowphol et al. 2015, McLeod et al. 2015). It is possible that different populations now assigned to *L. palavanensis*, even within Borneo, may turn out to be different species (Maximilian Dehling et al. unpublished).

## **Methods**

### *Study Area*

Fieldwork was conducted at the Kuala Belalong Field Studies Centre (KBFSC), a research facility at the heart of the Ulu Temburong National Park, Temburong District of the Sultanate of Brunei Darussalam. Ulu Temburong is the only national park in the country. This largely pristine forest is located on the Northwest coast of the island of Borneo and comprises fifty thousand hectares of lowland mixed dipterocarp rainforest. This forest is one of the tallest tropical forests in the world, with trees being 30–40 m tall, although individual trees can reach heights over 50 m. The Institute of Biodiversity and Environmental Research (IBER) and the Universiti Brunei Darussalam manage the KBFSC, located at 115° 09' E, 4° 33' N and about 50 – 200 m above sea level. The topography around the station is steep, with several slopes and with a loose layer of clay soil. There are no ponds or bodies of standing water except for ephemeral rain pools and

bearded-pig wallows. The mean temperature during the sampling time (1700h – 0000h) was  $25.2 \pm 1.9$  °C with a relative humidity of 90% (Goyes-Vallejos, pers. obs.). The yearly rainfall at the site varied between 4900 and 6800 mm without a well-defined dry season, although the driest period tends to be between June and August (KBFSC Weather Data 2005–2014). A population of *L. palavanensis* is found in the forest surrounding the research station. The study took place from June to July 2012, July to November 2013, and June to December 2014. The work on the vocal behavior of *L. palavanensis* reported here took place during the 2012 and 2013 field seasons.

### *Behavioral Observations*

Our study area was adjacent to the field station and encompassed approximately 1.3 ha of forest. The low density of understory vegetation allowed us to survey the area systematically making use of three main transects as our starting points and making a series of hairpin turns perpendicular to the transects. We surveyed this area almost every night (145 nights in total) searching for calling individuals of *L. palavanensis*. The acoustic encounter surveys began at 1700h and continued until 2300h and involved walking slowly along the main transects listening carefully for calls. It is nearly impossible to locate either males or females that are not calling, although a few were found through chance encounters. When a calling individual was found, we performed focal observations *ad libitum*. We recorded time of day, temperature, and number of individuals within a 2-m radius around the focal frog, along with other natural history notes. In addition to behavioral observations, whenever possible we made sound recordings of calling individuals for the acoustical analysis portion of this study. The

snout-urostyle length (SUL) of the focal individual was measured with a caliper (to the nearest 0.1 mm) and its mass obtained with a portable digital scale (to the nearest 0.01 g). We calculated a Body Condition Index using the residuals of the linear regression of SUL and mass. The tip of a toe of each front foot and hind foot were clipped for individual identification. To aid in the collection of natural history observations, a few individuals were taken to the laboratory at the research station and kept in captivity in a glass terrarium for one night. This study was done in compliance with the UConn – IACUC-approved protocol A12-028.

### *Sound Recordings*

We recorded 562 calls from 33 individual males and 26 females at night starting at 1700h until 2300h (mean temperature  $25.2 \pm 1.9$  °C, range: 22–28 °C) using a Marantz PMD 661 recorder (44.1 kHz sample rate, 16-bit resolution; Marantz America, LLC., Mahwah, NJ, USA) and a Sennheiser ME 66 directional microphone (Sennheiser USA, Old Lyme, CT, USA). In all cases the microphone was positioned 20–30 cm away from the calling individual. Sound pressure level measurements (db SPL re 20  $\mu$ Pa, C frequency weighting, fast response) were taken at a distance of 50 cm from each calling male using a digital sound level meter Extech 407730 (Extech Instruments, Waltham, MD). It was not possible to obtain the measurements of the sound pressure level of female calls due to their low amplitude and short duration. For every individual, we recorded whether calls were given in the presence or absence of other individuals, and the sex of any other individuals that were present.



### *Acoustical Analysis*

We used Raven Pro v1.4 software (Cornell Laboratory of Ornithology, Ithaca, NY, USA) to measure spectral properties such as dominant frequency, fundamental frequency, frequency modulation, number of harmonics and the harmonic of the dominant frequency. We defined harmonics as the spectral components appearing as integer multiples of the fundamental frequency, with the fundamental frequency being the first harmonic. We also measured temporal properties such as call duration, call rise time, and call fall time. The advertisement call of males of *L. palavanensis* is a trill (call with multiple sequential pulses), so we measured additional temporal properties that included pulses per call, pulse rate, pulse duration, pulse rise time, pulse fall time and interpulse interval. The spectrograms were generated using a 512-point fast Fourier transform (FFT) for male calls and 256-point FFT for the female calls.

All statistical analyses were conducted using R version 3.2.2 (R Core Team 2015). We report the mean and standard deviation for each measured acoustic property using a mixed effects linear model with frog ID as the random effect to correct for the fact that some individuals call more often than others, resulting in more calls to analyze for some individuals. For count data (number of harmonics, number of pulses) we report the median and interquartile range. We described the frequency modulation of the male advertisement call by plotting pulse number versus dominant frequency at each pulse. Then we fitted a third degree polynomial using a linear mixed effects model with individual frog as the random effect. We report coefficients of variation within ( $CV_w$ ) and among individuals ( $CV_a$ ) as percentages ( $CV = 100\% \times SD/mean$ ) using the standard deviation within individuals and among individuals from the model. We assessed the

relationship between dominant frequency and body mass, snout–urostyle length and body condition for both males and females. In addition, we assessed the relationship between male trill duration and mass, snout–urostyle length, and body condition. We tested for significance of the correlation fitted with a linear mixed effect model using a Kenward-Roger approximate F test. Because the temperature at our study site was fairly constant, we disregarded the effect of temperature on the properties of the calls.

### *Calling Rates of Males and Females*

In 2013, the study area surrounding the KBFSC was visited almost every night to locate calling individuals through acoustic encounter surveys (AES). We obtained these data concurrently while doing the sound recordings of calling individuals. When an individual was located, we would remain quiet to avoid disturbing the individual and registered the number of calls *ad libitum* until the individual stopped calling. We broke off the observation if the individual did not resume calling within 30 min. The number of calls of each individual per recording time period was standardized to number of calls per hour to calculate the average calling rate of males and females throughout the sampling period (1700h – 2300h). We fitted a generalized linear model predicting the number of calls as a function of sex (male, female). Because of the variable sample periods for each individual, we used an offset of the log number of minutes and fitted it as a quasipoisson distribution using a log link function.

## Results

### *Behavioral Observations*

From June 5 to July 24, 2012 and July 7 to November 20, 2013, we observed 44 females and 35 males of *L. palavanensis* (N = 79). The mean ( $\pm$ SD) snout–urostyle length (SUL) of adults was  $30.7 \pm 1.2$  mm for females (range: 27.9–33.1 mm) and  $26.4 \pm 1.6$  mm for males (range: 21.1–30.6 mm); females were significantly larger than males (Mann-Whitney test  $U = 25$ ,  $P < 0.001$ ). Besides size, there are no other sexually dimorphic morphological traits to distinguish males from females. However, it was evident that all calling females were gravid, because the white eggs filling the abdominal cavity were easily observed through the skin of their bellies.

Males of *L. palavanensis* were highly dispersed in the forest, with individual males separated by many meters ( $> 10$  m). The frogs called from the ground and did not use elevated perches, nor did they form aggregations or choruses. They do not call frequently, but their calls can be heard from 20 meters away by an observer. No more than four male frogs were found calling on a given night, and there were many nights on which males failed to call at all. There seemed to be more calling females than males each night, but due to the low intensity of their calls, females are hard to detect and their abundance probably is underestimated. We were able to find up to four calling females per night.

### *Calling Behavior of Males*

The advertisement call of *L. palavanensis* is a short trill (call with multiple sequential pulses) of moderate intensity (70 dB SPL at 50 cm). Over the course of a few

seconds before calling, the male inflates his whole body by inhaling air several times. At the end of this process, the flanks are visibly expanded. During calling, the whole body of the frog shakes fiercely for the duration of the trill. After each advertisement call, it can take from one minute to a full hour for a male to give another call. Their low calling rates and their cryptic coloration render the males very difficult to find.

### *Calling Behavior of Females*

Females called in response to the male advertisement call with a single-note squeak or chirp. We could hear the calls of an approaching female only when she was less than 3 m from the calling male. Once a female is within 1 m, the male sometimes responded to the female with a short courtship call (single note call) (Fig 4.2). This short male call was often given as an immediate response to the female call (response time mean =  $0.7 \pm 0.09$  s, median = 0.5 s, range = 0.2–2.2 s, N = 17) (Fig 4.3). Males did not approach the females and they did not give the courtship call in the absence of a female nearby. We observed 17 cases where more than one female was found calling around a male (within a 2 m radius). We also observed that females called antiphonally when more than one calling female was present, but we were unable to obtain recordings of these interactions due to the very low intensity of the female calls. Females also were found alone calling spontaneously (N = 21) at various times throughout the night in the absence of males or male advertisement calls. To confirm that females call spontaneously, 12 females were kept in the laboratory at the KBFSC field station in isolated conditions for one night. All 12 captive females called spontaneously at various times throughout the evening (1600h – 0000h). Descriptive statistics and coefficients of variation for the

spectral and temporal properties measured for all *L. palavanensis* calls are presented in Table 4.1.

#### *Male Advertisement Call*

We recorded 26 individuals giving the advertisement call, but only recorded the snout-urostyle length of 16 of them. The other males escaped immediately after the sound recording. The male advertisement call has an average duration of  $1899.3 \pm 267.2$  ms, with a dominant frequency of  $1888 \pm 81$  Hz and a fundamental frequency of  $611 \pm 22$  Hz. The median number of pulses per call was 28 pulses (interquartile range: 27–31 pulses), with the first pulse being significantly longer ( $77.8 \pm 10.7$  ms) and different in structure from the rest of the pulses ( $41.6 \pm 0.7$  ms) (paired *t* test:  $t_{59} = 5.14$ ,  $P < 0.0001$ ) (Table 4.2). The average pulse rate was 14 pulses/s (range: 10–17 pulses/s). The advertisement call had a median of six harmonics (interquartile range: 5–12), with the third harmonic being the dominant frequency. The call had an average upward frequency modulation between the 1<sup>st</sup> and 28<sup>th</sup> pulse of  $388 \pm 97$  Hz,  $N = 21$  (Fig 4.4).

#### *Female Call*

The female call is a short chirp, lasting on average  $56.8 \pm 9.0$  ms. The first harmonic is the dominant frequency, which makes the fundamental frequency and the dominant frequency the same ( $1608 \pm 67$  Hz). The median number of harmonics is eight (interquartile range: 4 – 15) (Fig 4.5).

### *Male Courtship Call*

Of the initial 33 males, only 17 were recorded giving the courtship call. This call was always given in response to a female call and is a soft single note squeak with an average dominant frequency of  $1752 \pm 86$  Hz and an average fundamental frequency of  $514 \pm 48$  Hz. The call has an average duration of  $309 \pm 126$  ms and consists of a single note, but there were instances in which the males gave two consecutive calls ( $N = 5$ ). In one exceptional case, a male gave the courtship call four consecutive times (Fig 4.6). The median number of harmonics was eight (interquartile range 5–13), with the third harmonic being the dominant frequency.

### *Call Variation*

We also explored the variation within and among individuals in the different spectral and temporal parameters of the calls. Differences in spectral and temporal properties of the call among individuals can influence mate choice by females (Gerhardt 1991). Following Gerhardt and Huber (2002), and Pettitt et al. (2013) we classified the acoustic parameters as ‘static’ if the values of  $CV_w$  were less than 7% and ‘dynamic’ if the values of  $CV_w$  are greater than 12%. In general, static acoustic properties are related to species recognition and are constrained by physical and physiological processes. The dynamic acoustic properties are more dependent on the social context that elicits the vocalization. Based on the  $CV_w$  values, dominant frequency and the fundamental frequency are static properties for the three types of vocalizations in *L. palavanensis*, while call duration, call rise time and call fall time are the dynamic properties based on the  $CV_w$  values. These results are similar to those observed in other frogs (Gerhardt and

Huber 2002) where there is low variation in general in the spectral properties due to the importance of frequency in species recognition. Within the pulse properties of the male advertisement call, pulse rate, interpulse duration, and pulse duration comprise the static properties, whereas the number of pulses per call and the duration of the first pulse are the dynamic properties. Overall, there is little variation among individuals in the values of dominant frequency and fundamental frequencies for all three of the call types and for the pulse rate in the male trill ( $CV_a < 10\%$ ). The greatest variation among individual is found in the temporal parameters for both male advertisement call and female call. In the male courtship call, there is more variation within individuals than among individuals.

#### *Correlation Between Acoustic Properties and Morphology*

Dominant frequency was not significantly correlated with mass, body size, or condition for either males or females (all  $P > 0.05$ ). Male advertisement call pulse rate was not correlated with any of the parameters tested. However, call duration was positively correlated with body size ( $F_{1, 12.8} = 5.09$ ,  $P < 0.05$ ) (Fig 4.7).

#### *Relative Calling Rates of Males and Females*

In 2013, we estimated the calling rates of 94 individuals (54 males, 40 females) throughout the evening over the course of five months. Females started calling at about 1700 h and continued to call throughout the evening (Fig 4.8). Males regularly started calling sporadically after sunset (1700h), but usually stopped calling by 2200h. Some males had calling rates as low as one call per hour ( $N = 3$ ). Females sometimes continued to call for up to an hour after males had ceased calling. Females also called sporadically

without a particular bout pattern, calling on average three times per minute, but they sometimes called up to five times per minute ( $N = 5$ ). Males had significantly lower calling rates than females throughout the evening ( $\beta_{\text{male number of calls}} = -3.3 \pm 0.2$ ,  $P < 0.001$ ). The predicted mean female calling rate was  $182 \pm 22$  (mean  $\pm$  SE) calls per hour, whereas the predicted mean male calling rate was  $6.5 \pm \text{SE } 1.1$  calls per hour.

## Discussion

Most aspects of the vocal behavior of species within *Limnonectes* remain unknown. There are very few descriptions of the male advertisement calls of most species and some of them are still considered voiceless (Emerson 1992) despite growing evidence of calling in species lacking vocal sacs (Matsui 1995, Rowley et al. 2014). The advertisement call of *L. palavanensis* is a moderately loud trill used in mate attraction. In contrast to other leaf-litter species with terrestrial egg deposition, which form clusters of calling males throughout the forest or near temporary ponds (Hauselberger and Alford 2005, Kaefer et al. 2012), males of *L. palavanensis* were not observed forming aggregations or choruses. Males of *L. palavanensis* called infrequently and on very few nights during the study period. Forest and leaf-litter species usually have low calling rates (Zimmerman and Bogart 1984), but the calling rate of *L. palavanensis* males is the lowest ever recorded.

We found that females not only call in response to a male advertisement call, but also initiate calling without a male acoustic stimulus. Female calling in *L. palavanensis* has not been previously reported. Spontaneous female calling has been observed also in *L. leporinus* (formerly *blythii*, Emerson, 1992), but it has not been reported in other



members of the genus. In general, female calls are usually very soft and hard to hear; therefore, their absence is more difficult to document than their presence (Wells 2007). Thus, it is possible that female calling is found in other species of *Limnonectes*. The calls of *L. palavanensis* females differ in temporal and spectral properties from the male advertisement call, being shorter and lower in intensity. Some hypotheses about the function of female calling include the idea that females initiate calling to aid in localization when males are widely dispersed in the breeding area (Given 1993). In *L. palavanensis*, males not only are scattered throughout the forest, but they also seem to be less abundant sex, considering that when a male gives an advertisement call, it can attract more than one female at a time, despite calling infrequently.

When one or more calling females approach a calling male, the male produces a different call as the females get closer. This vocalization was previously unknown and we describe it as a male courtship call, since it is given only as a response to the female call. In some species, males produce courtship calls, possibly to make themselves more conspicuous to a given female (Gerhardt and Huber 2002, Wells 2007). Females of the torrent frog *Odorrana tormota* elicit a distinct vocalization from the males, similar to what we observed in *L. palavanensis*. Males of *O. tormota* also exhibit precise phonotaxis toward female calls. Thus, this behavior seems to be more related to males being able to locate females in a noisy environment than to mate choice (Shen et al. 2008).

In *L. palavanensis*, the exact function of the male courtship call is not clear, although it is only elicited after a female has called. In species where both males and females are highly dispersed, female calling may have evolved to aid in location and

signaling of reproductive status (Bush, 1997). Female calling behavior may increase predation risk, because calling makes females more conspicuous; therefore, the trade-off has to have some compensatory benefits, e.g., increasing a female's ability to locate a high quality mate. In *L. palavanensis*, males do not always respond to a female call, but when they do, they respond within a narrow window of time. So, spontaneous female calling may aid in male localization if it successfully elicits male courtship calling.

When more than one female approaches a male *L. palavanensis*, an initial female call causes other females to start calling antiphonally. In males of the poison dart frog *Allobates femoralis*, antiphonal calling behavior is a sign of competition among males, and usually precedes physical aggression (Roithmair 1994). While we did not observe physical contact among females of *L. palavanensis*, females eavesdrop on other calling females approaching a nearby male and start calling themselves. This form of acoustic competition has also been observed in *Clinotarsus curtipes* (formerly *Rana curtipes*), where females compete with each other for mating opportunities when males are scarce (Krishna and Krishna 2005).

Advertisement calls signal to competitors to keep away while simultaneously attracting mates (Wells 1977). The term “female advertisement call” has been proposed before in other anurans (Given 1993, Tobias et al. 1998, Toledo et al. 2014). In *L. palavanensis*, the female call is serving the dual function of eliciting a response from the male while simultaneously alerting other females of their presence. However, we refrain from using the term advertisement call for females of *L. palavanensis* since we do not have experimental evidence of the function of this call in mate attraction or male mate choice.

Reports of female calling in frogs have increased recently (Boistel and Sueur 2002, Toledo et al. 2014). However, to the best of our knowledge, this is the first instance of an anuran species in which female calling rates dramatically exceed male advertisement calling rates. In birds, only in a few species do females commonly sing more often than males. In the Streak-backed Oriole (*Icterus pustulatus*), females call more often than males, probably as an aggressive signal while defending territories (Price et al. 2008). In the sex-role reversed African black coucal (*Centropus grillii*), females use vocalizations to defend large territories that encompass smaller male territories (Goymann et al. 2004). Females of *L. palavanensis* do not attract males to territories.

In *L. palavanensis*, females call spontaneously and more frequently than the males. Moreover, females engage in antiphonal behavior with neighboring females, and the males are the ones to respond rapidly with a specific courtship call. In contrast, males of *L. palavanensis* rarely call, and carry out all the parental duties while females desert the oviposition site after fertilization. Males guard the eggs for about ten days until hatching and transport the tadpoles to water (Chapter 2). These aspects of the reproductive behavior of *L. palavanensis* are consistent with a sex-role reversed mating system.

The mating system of a species is determined in part by which sex is the limiting resource. Therefore, individual tactics for acquiring mates will be influenced by the number of individuals available for mating at a given time (Emlen and Oring 1977). In *L. palavanensis*, males are spread throughout the forest and do not call very often, making them hard for females to locate. In addition, caring for the offspring effectively removes males from the mating pool (as no male was observed to care for more than one clutch at

a time), further reducing the probability of females to find males ready to mate. These aspects of the male calling behavior and the paternal behavior of this species may have facilitated the evolution of female calling. Multiple females approaching a male in a female-biased situation ultimately should lead to competition among females. Thus the evolution of high female calling rates should be favored if it increases the chances of obtaining a mate. It is still unclear, however, if the male eventually moves toward a female, or if the female moves towards the male, guided by the courtship calls given by males in response to female calls. Future work is necessary to investigate the function, if any, of female *L. palavanensis* calls in mate attraction.

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Figure 4.1. An adult female of *Limnonectes palavanensis* from Brunei Darussalam. Photo by Johana Goyes-Vallejos.

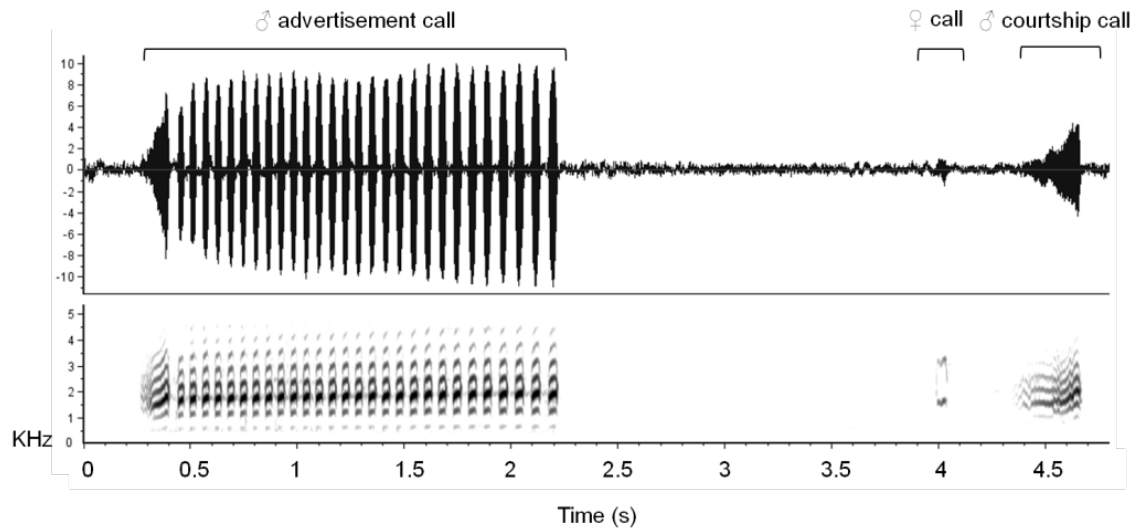


Figure 4.2. Oscillograms (top) and spectrograms (bottom) of the male advertisement call, female call and male courtship call of *L. palavanensis*. Males respond with a courtship call immediately after a female call. All three calls are represented in real time. Note the difference in amplitude between the male advertisement call, the female call and the male courtship call.

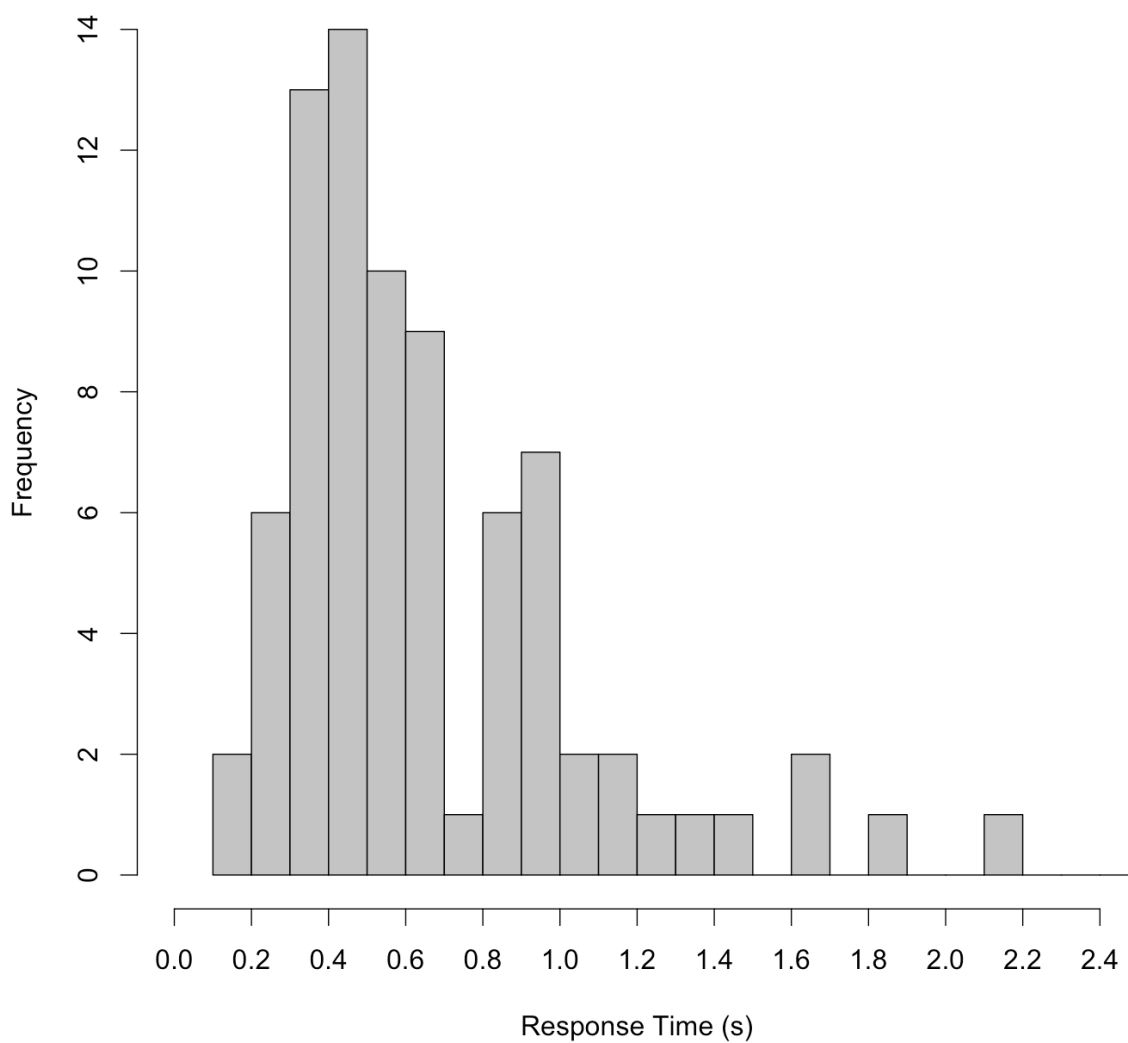


Figure 4.3. Histogram of the response times after a female call of *Limnonectes palavanensis* male courtship calls (N = 17, median = 0.5, range = 0.2–2.2 seconds).

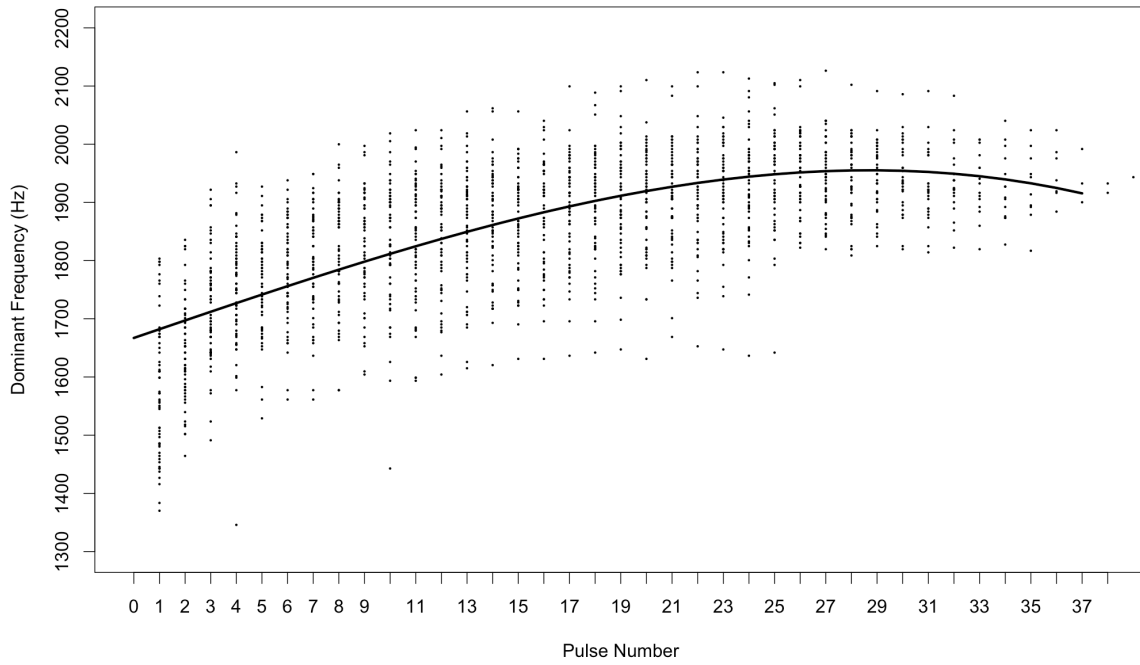


Figure 4.4. Upward frequency modulation of the male advertisement call of *Limnonectes palavanensis*. Fitted line was obtained by fitting a third degree polynomial using a linear mixed effects model with individual frog as the random effect (mean  $\pm$ SD frequency modulation of  $388 \pm 97$  Hz,  $N = 21$ ).

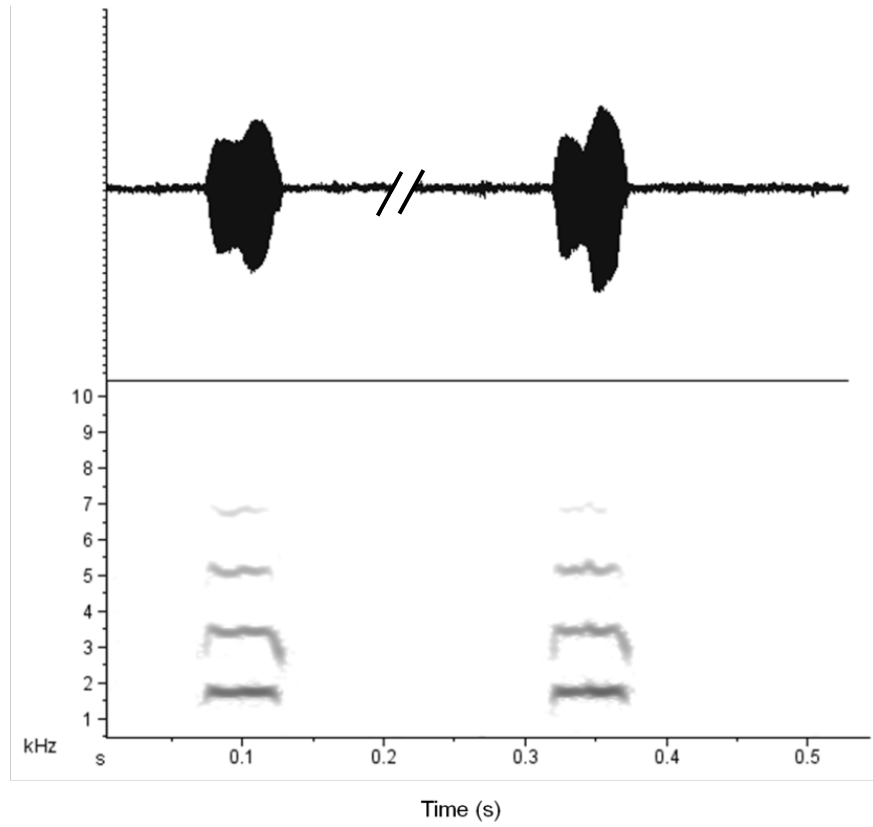


Figure 4.5. An oscillogram (top) and spectrogram (bottom) showing two calls of the same female *Limnonectes palavanensis*.

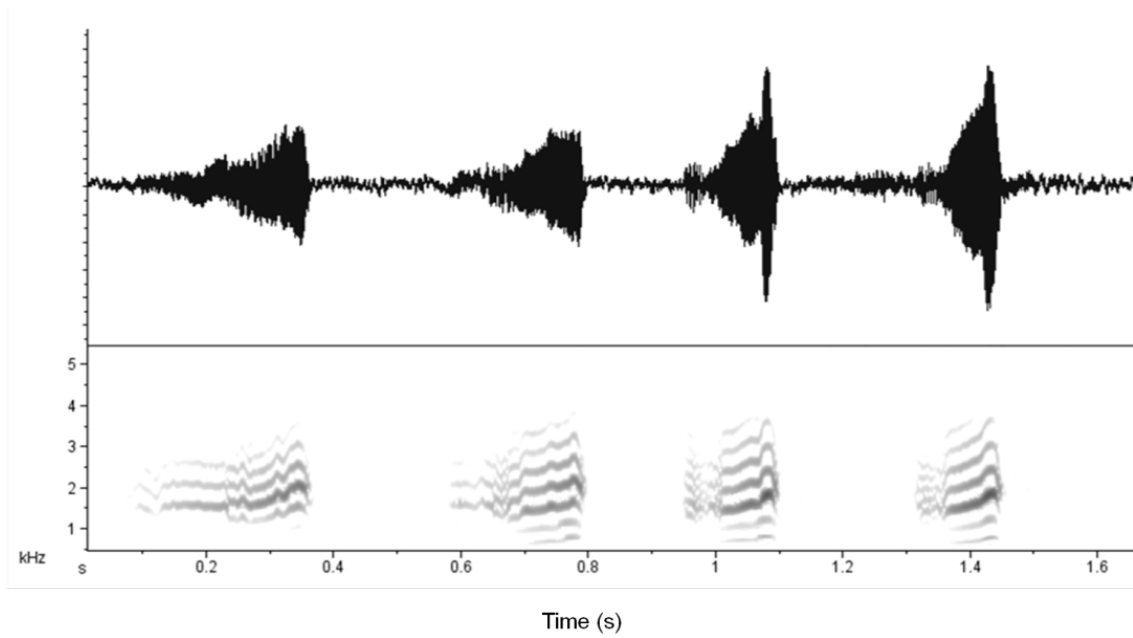


Figure 4.6. An oscillogram (top) and spectrogram (bottom) of four consecutive courtship calls from a single male *L. palavanensis* given as a response to a single female calling (not shown).

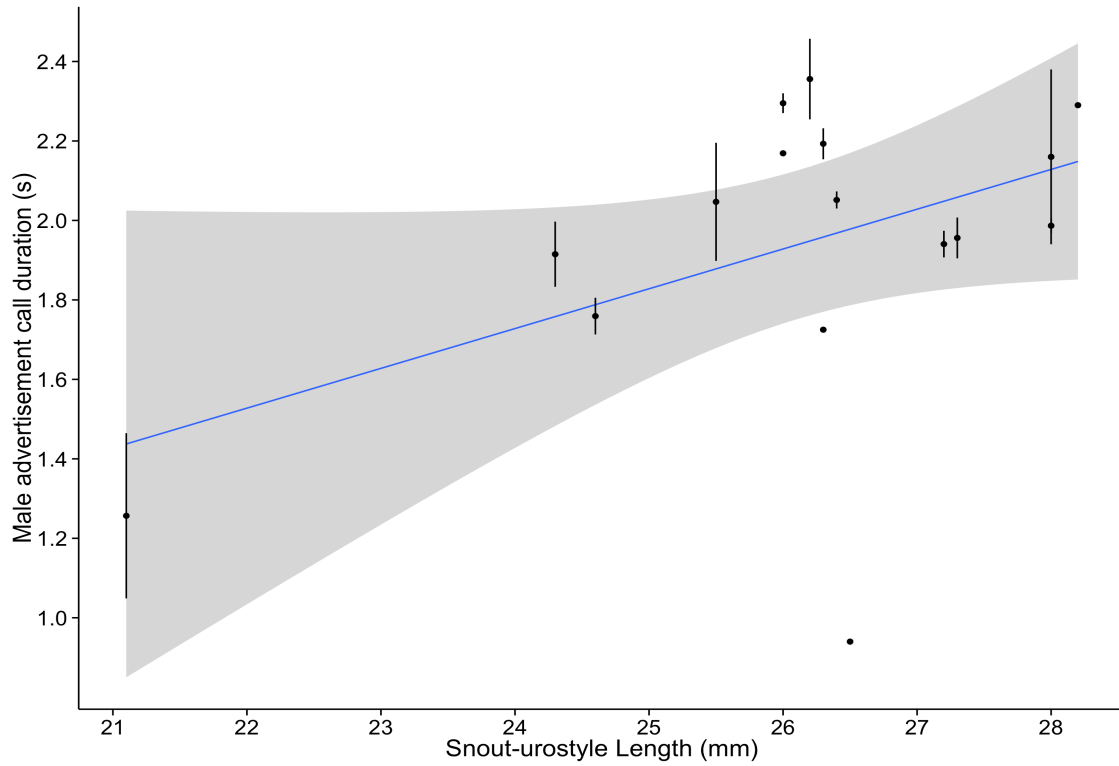


Figure 4.7. Correlation between advertisement call duration of male *L. palavanensis* and their snout-urostyle length. There is a positive correlation between call duration and body size in males ( $N = 16$ ,  $F_{1, 12.8} = 5.09$ ,  $P < 0.05$ ). Confidence intervals (95%) are shown in grey.



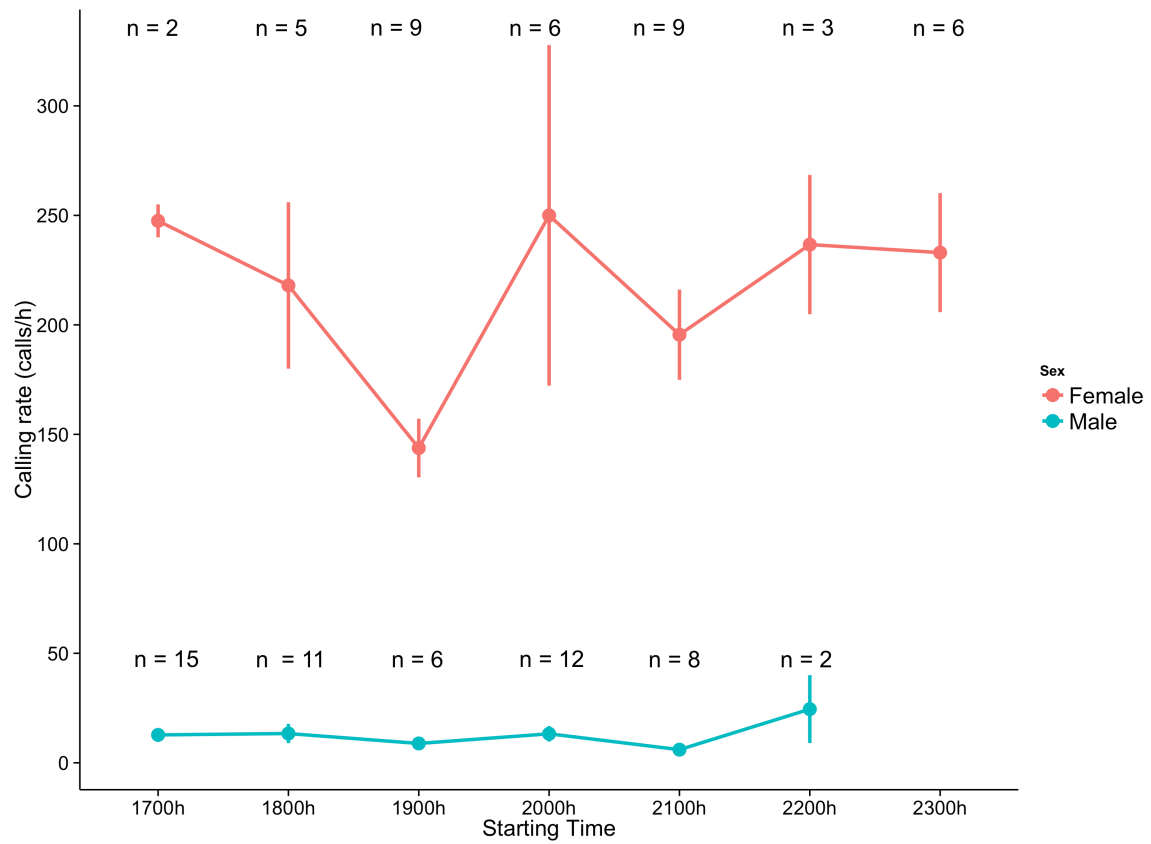


Figure 4.8. Average hourly advertisement calling rates of male and female *Limnonectes palavanensis* individuals based on focal observations of 54 males and 40 females (*n* represents the number of individuals sampled per time period). Bars reflect  $\pm$  standard error.

Table 4.1. Description of temporal and spectral properties measured for the three different calls of *Limnonectes palavanensis*

Acoustic Property	Male advertisement call				Female call				Male courtship call			
	$\bar{x} \pm SD$	Min – Max	$CV_w$	$CV_a$	$\bar{x} \pm SD$	Min – Max	$CV_w$	$CV_a$	$\bar{x} \pm SD$	Min – Max	$CV_w$	$CV_a$
<i>Spectral parameters</i>												
Dominant Frequency (Hz)	1888 $\pm 81$	1604 – 2062	3.2	4.3	1608 $\pm 67$	1378 – 1787	2.6	4.2	1753 $\pm 86$	1464 – 2143	7.1	4.9
Fundamental Frequency (Hz)	611 $\pm 22$	569 – 660	1.9	3.7	1608 $\pm 67$	1378 – 1787	2.6	4.2	514 $\pm 47$	379 – 624	8.0	9.2
<i>Temporal parameters</i>												
Call duration (ms)	1899.3 $\pm 267.0$	773 – 2690.8	13.8	14.2	56.8 $\pm 9.0$	34 – 99	11.6	15.8	309.8 $\pm 126.0$	120 – 1146	46.5	40.7
Call rise time (ms)	812.3 $\pm 284.0$	225 – 1733	37.1	35.0	32.3 $\pm 6.7$	5 – 55	17.8	20.7	239.4 $\pm 67.8$	43 – 510	28.0	28.3
Call fall time (ms)	1074.4 $\pm 242.0$	222 – 2107	34.0	22.5	24.5 $\pm 6.2$	13 – 66	28.2	25.4	40.6 $\pm 17.8$	10 – 316	97.2	43.8

Table 4.2. Pulse temporal properties in *Limnonectes palavanensis* male advertisement calls.

<b>Temporal parameter</b>	<b><math>\bar{x} \pm SD</math></b>	<b>Min – Max</b>	<b>CV<sub>w</sub></b>	<b>CV<sub>a</sub></b>
Pulses per call (k)	28 (27 – 31) <sup>a</sup>	9 – 38	15.7	15.6
Pulse rate (pulses/s)	14 $\pm$ 1.2	10.3 – 17.1	5.7	8.6
1 <sup>st</sup> . Pulse duration (ms)	77.8 $\pm$ 42.4	29 – 349	48.7	54.5
1 <sup>st</sup> . Pulse rise time (ms)	60.2 $\pm$ 44.5	18 – 320	58.1	73.9
1 <sup>st</sup> . Pulse fall time (ms)	19.6 $\pm$ 4.5	9 – 33	22.8	22.8
Interpulse duration (ms)	25.6 $\pm$ 4.0	19.1 – 40.1	5.3	15.6
Pulse duration (ms)	41.7 $\pm$ 3.5	34.3 – 48.7	2.5	8.4
Pulse rise time (ms)	19.2 $\pm$ 3.0	13.6 – 27.8	6.9	15.7
Pulse fall time (ms)	22.2 $\pm$ 3.1	15.1 – 30.7	7.5	14.0

<sup>a</sup> For pulses per call we report the median and the interquartile range.

**CHAPTER 5. FUNCTIONAL SIGNIFICANCE OF MALE AND FEMALE CALLING IN THE  
SMOOTH GUARDIAN FROG *LIMNONECTES PALAVANENSIS*: EVIDENCE FROM PLAYBACK  
EXPERIMENTS**

**Introduction**

In most animals, females invest more in their offspring than do males. In these conventional systems, sexual selection typically acts more strongly on males than on females, resulting in male-male competition for access to females. This makes females choosy (Kokko and Jennions 2008, Alonzo 2010). In sex-role reversed systems, males carry out all parental care duties, foregoing additional mating opportunities. Thus, females compete for males, as they are now the limiting resource and consequently the choosy sex (Trivers 1972, Vincent 1992).

In vertebrates, cases of sex–role reversal have been well documented in fishes (sea horses and pipefishes) and birds (Vincent et al. 1992, Emlen et al. 1998, Eens and Pinxten 2000). In the African black coucal *Centropus grillii*, males mate with only one female throughout a given breeding season and provide exclusive parental care, while females can mate with up to four males. Females vocalize more vigorously than males and use these vocalizations to defend large territories encompassing several small male territories (Goymann et al. 2004, Voigt and Goymann 2007). Playback experiments with female calls elicited changes in the frequency, composition and duration of the calls from females (Geberzahn et al. 2009). These results suggested that this species has intrasexual competition among females and that females are capable of assessing body size of potential competitors.

In frogs, calling by females is rare, but does occur in some species, usually when females produce short, soft courtship calls in response to the advertisement calls of males (Wells 2007). This sometimes stimulates reciprocal calling by males, which may provide directional cues to females searching for calling males (Bush et al. 1996, Tobias et al. 1998, Bosch 2001). A few species exhibit at least partial reversal of the usual sex roles in calling behavior. For example, in the Majorcan midwife toad (*Alytes muletensis*), behavioral studies in the laboratory showed that females physically compete for access to the males by tackling other females or pushing a male away from an amplexant pair. (Bush and Bell 1997). In the Chinese concave-eared torrent frog (*Odorrana tormota*), males and females assemble in groups on rocks in noisy streams, and individuals of both sexes call to each other with distinctively different calls. In the field and in playback experiments, males exhibited precise phonotaxis toward the calls of females, the reverse of the usual pattern of females approaching calling males (Shen et al. 2008).

We used playback experiments of both male and female calls to investigate the function of vocal signals in a frog that exhibits some elements of sex-role reversal. The smooth guardian frog (*Limnonectes palavanensis*) is a small leaf litter frog (males SUL = 21.1–30.6 mm, females SUL = 27.9–33.1 mm) found on the island of Borneo. In this species, males perform all parental duties, from attending the eggs to transporting the tadpoles to small bodies of water where they finish their development (Inger et al. 1986, Inger and Voris 1988, Chapter 2). Males produce two call types: a long advertisement call and a short courtship call. Females produce a short, soft courtship call, not only in response to male calls, but also spontaneously in the absence of calling males (Chapter 4). With its male parental care and female vocalizations, *L. palavanensis* offers a unique

opportunity to test predictions of sexual selection theory. In a sex–role reversed system, males are expected to be a limiting resource for females, and male–male competition is expected to be low. On the other hand, if receptive females outnumber receptive males, females are expected to compete for access to males, either through physical interactions or exchange of signals (Trivers 1972, Summers 1989, Summers and Tumulty 2013).

Using a protocol similar to that used in studies of the sex–role reversed black coucal (Geberzahn et al. 2009), we used field playback experiments to test the response of both males and females to all call types of both sexes. We predicted the following outcomes: (1) If males engage in aggressive competition or territorial behavior, then males should respond to the playbacks of male advertisement calls with advertisement calls of their own (this species does not have a distinct aggressive call). (2) Males are expected to respond to playbacks of female calls by switching to short courtship calls, as observed in the field (Chapter 4). (3) Females should respond to both male advertisement calls and male courtship calls with calls of their own. (4) Females should respond to playbacks of female calls by increasing their own calling rates, if females are aggressive towards other calling females.

## **Methods**

We studied a population of the smooth guardian frog (*Limnonectes palavanensis*) (Family: Dicroglossidae) found in the rainforest adjacent to the Kuala Belalong Field Studies Centre (KBFSC). The KBFSC is located in the Ulu Temburong National Park (UTNP), Temburong District of the Sultanate of Brunei Darussalam (115° 09' E, 4° 33' N, 50–200 m altitude). The UTNP comprises fifty thousand hectares of lowland mixed

dipterocarp rainforest. The study was conducted during the 2013 field season (July–November) and the 2014 field season (August–December). All surveys began at 1700h and continued until 2300h and involved walking slowly through the study area (ca. 1.3 ha.) listening carefully for calls of males and females. Whenever possible, every individual was measured (snout-urostyle length to the nearest 0.1 mm) using a caliper, and given a unique identification number by clipping the tip of a toe of each front foot and hind foot.

### *Field Playback Experiments*

The sound stimuli used for the playback experiments were recordings of natural male and female calls obtained during the 2013 field season. We used five exemplars for each type of call with the average duration and dominant frequency found in the population; exemplars were rotated among playback experiments. After locating a calling individual in the field, we set up two speakers (SME–AFS Amplified Playback Speaker, Saul Mineroff Electronics, Inc. Elmont, NY) one meter away from the focal individual. The speakers were ca. 20 cm from each other. One of the speakers broadcast the male advertisement call or the male courtship call and the other speaker broadcast the female call, to simulate the male and the female calling in close proximity (Fig 5.1). The specific stimulus for each test was played at a rate of 1 call/min. We performed one set of playback experiments for males and one set for females.

We tested 16 focal males. The playback experiment for the males consisted of six 10 min test periods. The first test was the control, when no playback was broadcast. The playback stimuli were (1) a male advertisement call, (2) a male advertisement call

followed by a female call 0.5 s later, (3) a male courtship call, (4) a female call, and (5) a female call followed by a male courtship call 1 s later. The presentation order of the tests was randomized for each test subject.

We tested ten females of *L. palavanensis*. Each female was presented with five 6-min test periods. It was not possible to obtain control recordings of spontaneous calling by the females, because they usually stopped calling when found and did not resume calling. We used the same series of playback tests described for the males. The presentation order of each test was randomized.

For both male and female playback experiments, there was an initial silent period of 10 minutes before initiating the first test, and 10 minutes between tests. The responses of the focal individuals were recorded using a Sennheiser ME66 directional microphone (Sennheiser USA, Old Lyme, CT, USA) and a digital recorder (Marantz PMD661) (44.1 kHz sample rate, 16-bit resolution; Marantz America, LLC, Mahwah, NJ, USA).

### *Data Analysis*

We assessed the number of advertisement calls and courtship calls given by males and the number of calls given by females from the recordings obtained during each of the playback tests. To test for differences in the number of calls among the playback tests, we used linear mixed effects models using the *lme4* package (Bates et al. 2015) in R version 3.2.3 (R Development Core Team 2015). The number of calls was modeled as a function of the playback test using a Poisson distribution and individual frogs as a random effect to control for individual variation in calling rates.



## Results

### *Playback Experiments with Males*

During the control recordings, the median number of calls during 10 minutes was three (range = 1–7) (Table 5.1). Males significantly increased the number of advertisement calls during the playback of the male advertisement ( $\beta_{\text{male.adv}} = 0.5 \pm 0.2$ ,  $P < 0.05$ ) and during the male advertisement call + female call test ( $\beta_{\text{male.adv+female}} = 0.6 \pm 0.2$ ,  $P < 0.001$ ) compared with the control. However, the difference between these two treatment was not significant ( $P = 0.387$ ; Fig 5.2). While statistically significant, the responses of most test subjects to male advertisement calls were not dramatic, largely because of the inherently low calling rate of males. Nine of 16 males gave more calls during playbacks of the male advertisement call than during the control period. Ten of 16 males gave more calls during the playback of the male advertisement + female call than during the control period (Table 5.1).

Test males did not respond with advertisement calls to the female call, the male courtship call, or a combination of the two (Fig. 5.2), and none of these tests was significantly different from the control ( $P > 0.05$ ). Only two individuals gave any advertisement calls in response to the female call alone (Table 5.1).

The previous playback experiments indicated that males engage in limited vocal competition with other males by increasing the rate of advertisement calling, although calling rates of most males remained low (Table 5.1). Males also appear to compete by overlapping their own advertisement calls with the broadcast call. Nine of the 16 focal males produced advertisement calls overlapping the playback stimulus during the male advertisement call test, and the male advertisement call + female call (Fig 5.3). This

occurred more frequently when the playback of a male advertisement call was coupled with a female response ( $\beta_{\text{male.adv+female}} = 1.1 \pm 0.3$ ,  $P < 0.001$ ; Fig 5.4).

Males showed an increase in their courtship call rate in all the playback tests including a female call. During the playback of the male advertisement call + female call, males increased the number of courtship calls produced ( $\beta_{\text{male.adv+female}} = 2.1 \pm 0.4$ ,  $P < 0.001$ ). Males also significantly increased the number of courtship calls when presented with a playback of the female call alone ( $\beta_{\text{female}} = 3.3 \pm 0.4$ ,  $P < 0.001$ ) and the female call + male courtship call ( $\beta_{\text{male.courtship+female}} = 2.8 \pm 0.4$ ,  $P < 0.001$ ) compared with the control. Males did not produce courtship calls during the control recording or during the playback of the male courtship call, and the number of courtship calls produced during the male advertisement call test was not significantly different from the control ( $P > 0.08$ ; Fig 5.5).

#### *Female Playback Experiments*

Females significantly increased the number of calls during the male advertisement call test, and the male advertisement call + female call test compared with a playback of the female call alone (both  $\beta = 0.7 \pm 0.2$ ,  $P < 0.001$ ; Fig 5.6). Likewise, females increased the number of calls during the male courtship call test ( $\beta_{\text{male.courtship}} = 0.7 \pm 0.2$ ,  $P < 0.001$ ), and the female + male courtship call test ( $\beta_{\text{female+male.courtship}} = 0.6 \pm 0.2$ ,  $P < 0.01$ ; Fig 5.6).

#### **Discussion**

Our study showed that males of *L. palavanensis* increase the number of advertisement calls produced when there is a simulated male producing advertisement

calls, but the overall rate of calling remained low in all tests. In some species of frogs, males hold territories where they can defend resources such as access to females, oviposition sites, food and shelter (Narins et al. 2003, Pröhl 2005). Males can deter other males from entering their territories by increasing rates of advertisement calling, producing aggressive calls, or physically attacking the opponent (Wells 2007). We found no evidence of territoriality in *L. palavanensis*, a result consistent with the wide dispersion of males across the forest floor (Chapter 4). Males did not engage in antiphonal calling behavior (alternation of calls), as observed in other frogs (Wells 2007), did not produce specific aggressive calls, and did not attack the speaker, as observed in males of the bullfrog *Lithobates catesbeiana* (Bee 2002) and in males of the poison-dart frog *Allobates femoralis* (Ringler et al. 2011).

We did observe one form of possible acoustic interference between males, especially when a simulated calling female was present. Males almost completely overlapped their own advertisement calls with the playback of an advertisement call. This type of apparently deliberate acoustic masking of a competitor's call has been observed in other frogs as well. In the Panamanian hylid frog *Dendropsophus ebraccatus*, which has a complex multi-note call, males respond to other males' calls by overlapping the long introductory note of their own calls with the short secondary click notes of a neighbor, thereby masking the click notes of the leading male. Females have been shown to prefer the following (masking) male's calls in playback experiments (Wells and Schwartz 1984). In the North American bird-voiced treefrog (*Hyla avivoca*), males overlapped an advertisement call stimulus by making their own calls longer; females preferred the calls of the masking male (Martínez-Rivera and Gerhardt 2008).

Males of many species of frogs produce short-range courtship calls in response to approaching females or to the calls of females. Usually these courtship calls are softer and shorter than advertisement calls, presumably to avoid alerting other males to the presence of a female (Wells 2007). Often males and females exchange courtship calls in a reciprocal fashion. Field observations of *L. palavanensis* showed that males give short courtship calls in response to the calls of females (Chapter 3). These observations were confirmed by our playback experiments; only those stimuli that included a female call (female call alone, male advertisement call + female call, female call + male courtship call) elicited courtship calls from test males.

Females of *L. palavanensis* gave vocal responses to all stimuli, but gave significantly more responses to stimuli that included a male advertisement or courtship call, either alone or in conjunction with a female call. This supports the role of the female call as a courtship call aimed at males. In nature, males are widely dispersed in the forest and call infrequently to attract females. Females call and males respond with their own courtship calls, and this exchange of short calls presumably facilitates location of potential mates. We do not know whether females approach males or males approach females, because a complete courtship sequence has not been observed. Field observations have shown that females also call spontaneously in the absence of males, presumably because males are difficult to locate, and female calling elicits courtship calling from males. Females also call considerably more frequently than males in the field (Chapter 4). These two observations are consistent with the predictions of the sex-role reversal hypothesis and provide evidence for reversal of the usual sex roles in anuran courtship.

One prediction of the sex-role reversal hypothesis is that females should engage in acoustic or physical competition with other females. For most stimuli used in the playback tests, females tended to give more responses than did males (compare Tables 5.1 and 5.2). However, this was least evident in female responses to female calls alone. Therefore, the playback experiments do not provide strong evidence for extensive vocal competition among females, nor do they support the idea that female calls serve as aggressive signals directed at other females. Similar results were obtained in a study of the Iberian midwife toad (*Alytes cisternasii*); females responded least to female calls, and the authors concluded that female calls were mainly directed at males and did not serve as aggressive signals directed at other females (Bosch 2002).

In conclusion, the intensity of intrasexual competition among females is not stronger than that of the males when measured in terms of differences in calling rates. In *L. palavanensis* males may become the limiting resource due to the prolonged parental care. Thus, in a sex-role reversed mating system, it is expected that females are more aggressive because there are competing for access to the few males available for mating. The results from our playback experiments with the females do not support the female-female competition prediction. However, the sex-role reversal hypothesis also states that males can become the choosy sex when there is an overabundance of females (Chapter 4). It is possible then, that mate choice by the males plays a more important role than intrasexual competition through acoustic signaling among females. Yet, additional aspects of the courtship behavior and how mate choice is achieved in this species are necessary to test further predictions of the sex-role reversal hypothesis.

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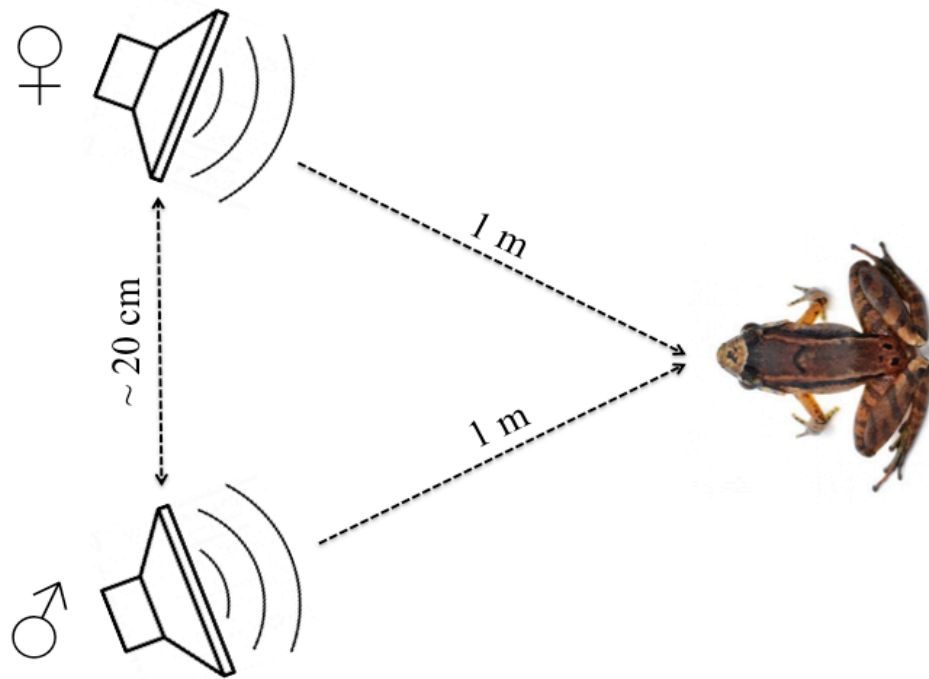


Figure 5.1. Schematic representation of the setup for the playback experiments. The two speakers were located ca. 20 cm apart and 1 m from the focal individual. One of the speakers broadcasted the male calls (advertisement call and/or courtship call) and the other speaker broadcasted the female call.

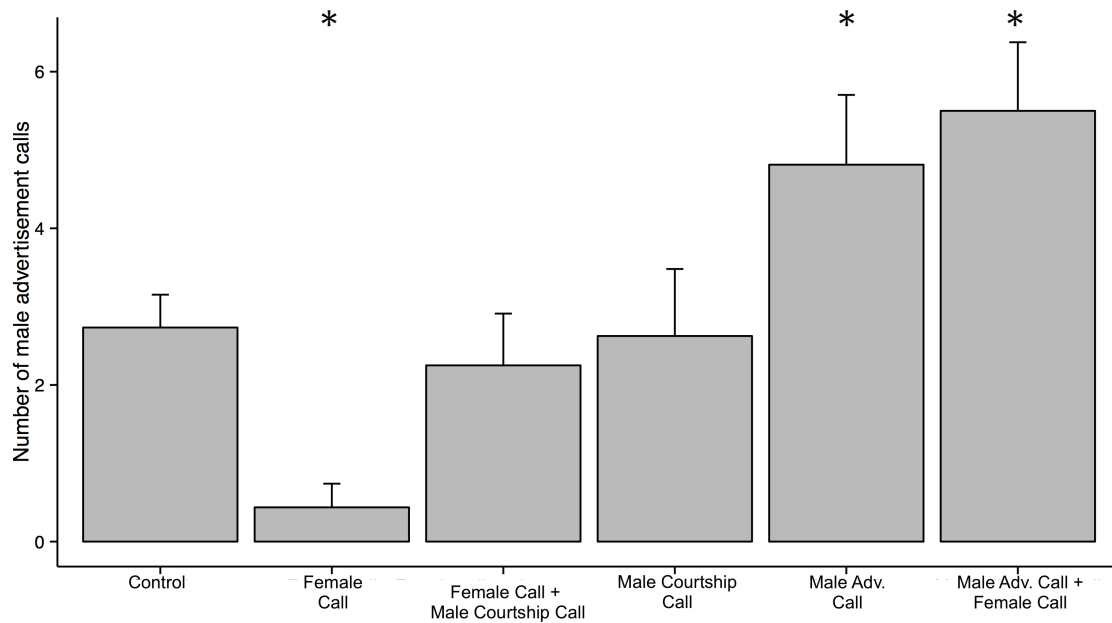


Figure 5.2. Playback experiments with males. Mean  $\pm$ SE number of advertisement calls produced by males of *L. palavanensis* recorded during five different 10-min playback tests (N = 16 males). The asterisks indicate significant differences from the control at the  $P < 0.05$  level.

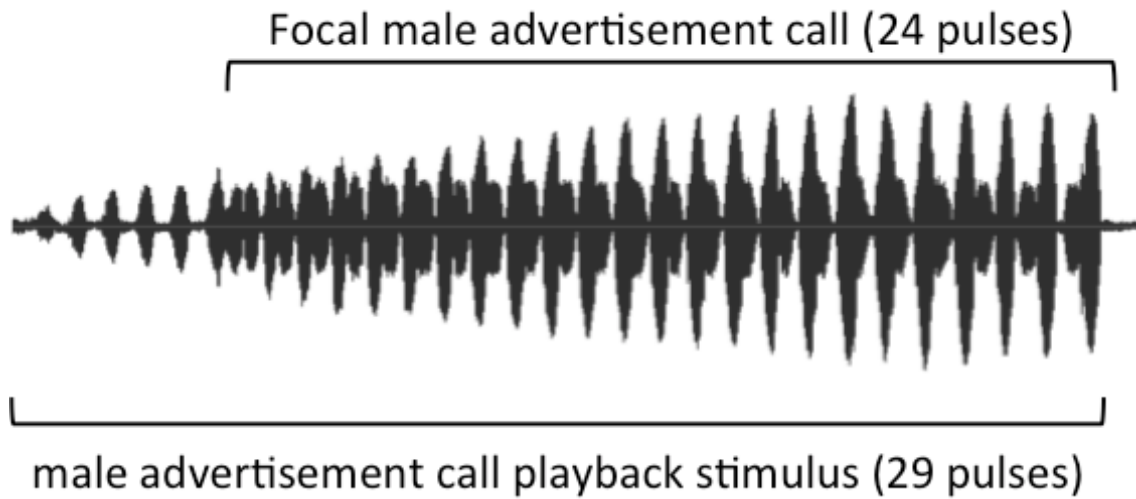


Figure 5.3. Oscillogram of a male *L. palavanensis* overlapping the playback stimulus of the male advertisement call with an advertisement call of his own. In this particular example, the focal male starts the call after the sixth pulse of the playback stimulus and ends exactly at the same time.

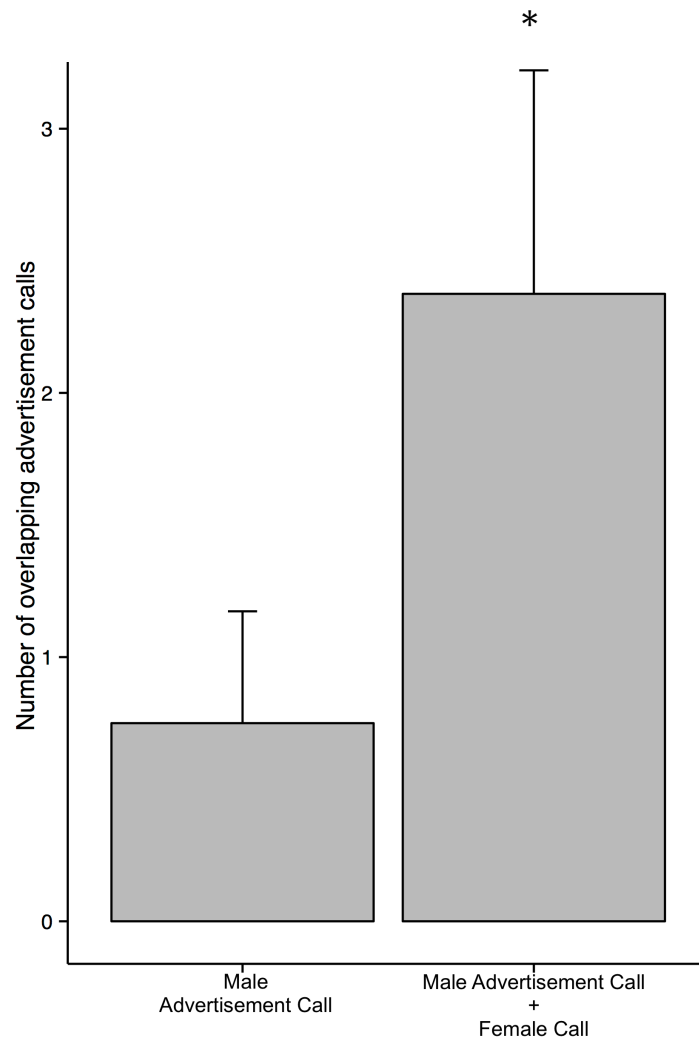


Figure 5.4. Playback experiments with males. Mean  $\pm$ SE number of advertisement calls produced by males of *L. palavanensis* overlapping the male advertisement call playback during the male advertisement call tests, and the male advertisement call + female call test (N = 9 males). The asterisks indicate significant differences at the  $P < 0.05$  level.

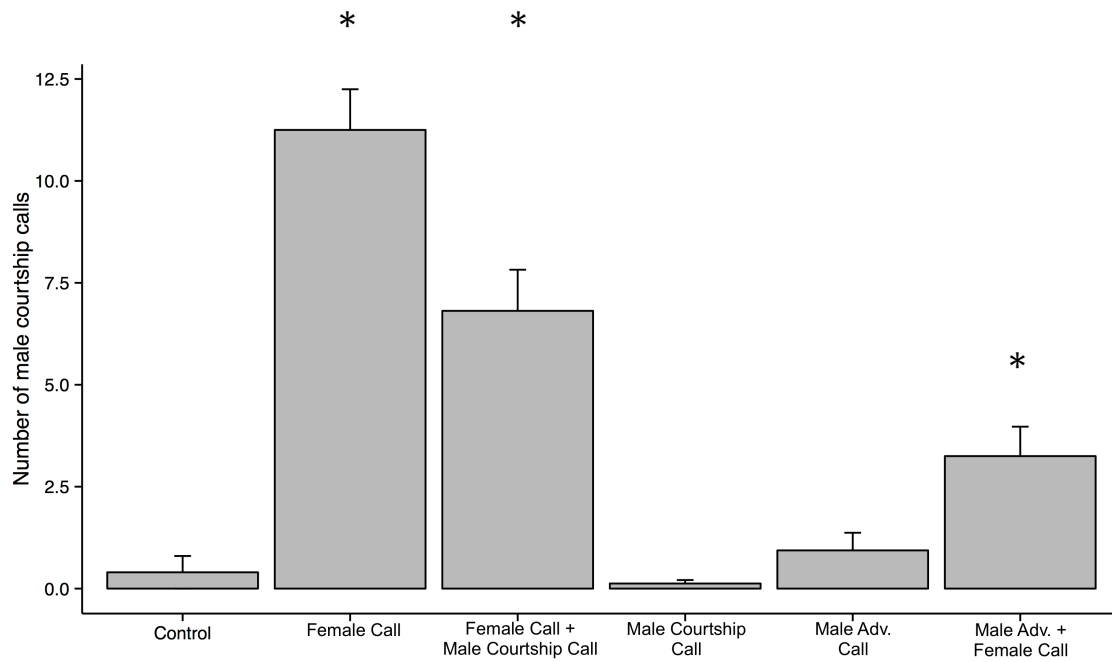


Figure 5.5. Mean  $\pm$ SE number of courtship calls produced by males of *L. palavanensis* recorded during five different 10-min playback tests (N = 16 males). The asterisks indicate significant differences from the control at the  $P < 0.05$  level.

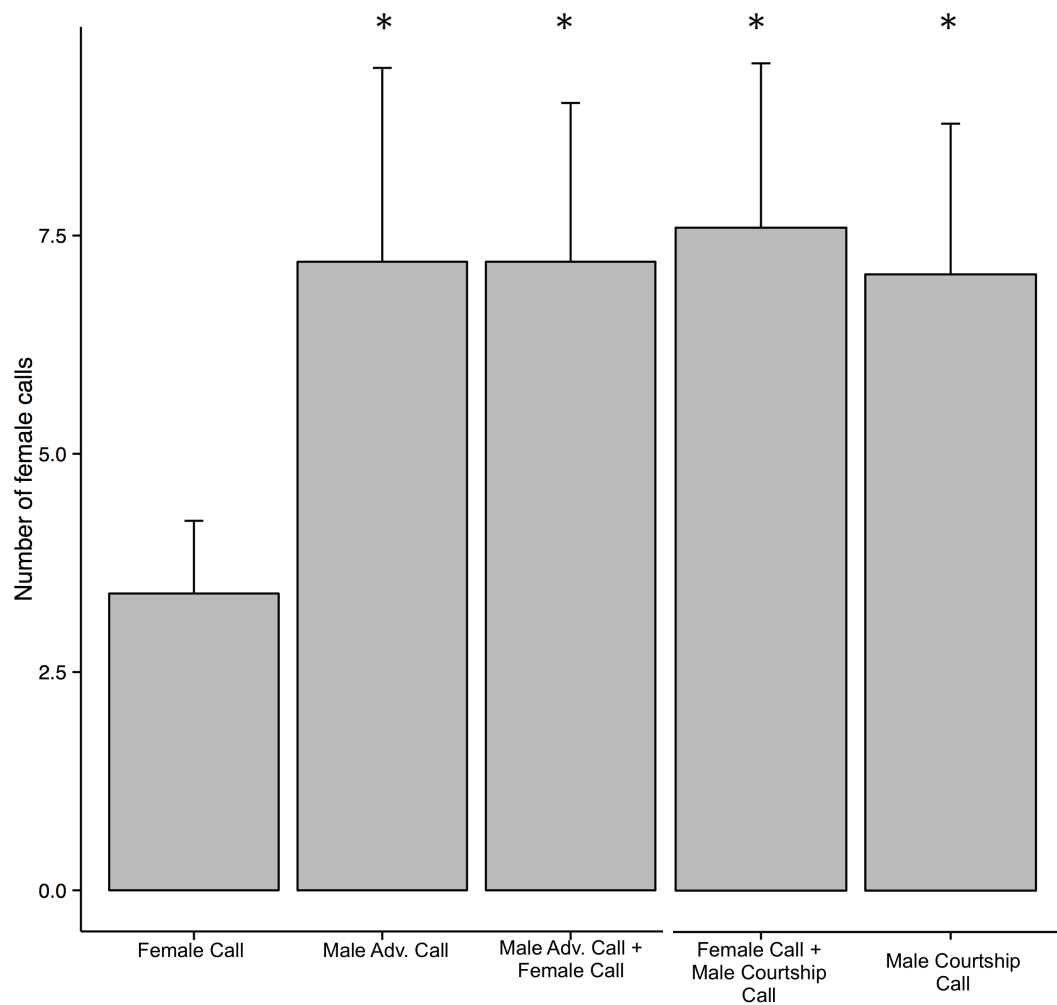


Figure 5.6. Number of calls (mean  $\pm$ SE) produced by females of *L. palavanensis* recorded during a playback of the female call, a male advertisement call, a male advertisement call + female call, a male courtship call, and a female call + male courtship call (N = 10 females). The asterisks indicate significant differences from the female call only playback test at the  $P < 0.05$  level.

Table 5.1. Playback experiments with males of *Limnonectes palavanensis*. Number of male advertisement calls produced per playback test (N = 16).

Ind.#	Male Advertisement Call				Female Call	
	Control	Male Advertisement Call	+	Male Courtship Call	+	Female Call
			Female Call		Male Courtship Call	
1	1	3	3	3	0	0
2	1	1	10	7	3	0
3	2	0	2	0	2	4
4	2	2	8	0	2	0
5	2	4	8	0	5	3
6	1	3	9	1	0	0
7	NA	8	7	10	10	0
8	3	2	1	0	0	0
9	3	3	4	0	1	0
10	3	9	9	1	0	0
11	1	6	7	8	3	0
12	3	10	11	5	3	0
13	4	8	3	6	3	0
14	4	4	0	0	0	0
15	4	2	4	0	0	0
16	7	12	2	1	4	0

Table 5.2. Playback experiments with males of *Limnonectes palavanensis*. Number of male courtship calls produced per playback test (N = 16).

Ind. #	Control	Male Advertisement Call			Female Call		
		Male Advertisement Call	+	Male Courtship Call	+	Female Call	Female Call
			Female Call		Male Courtship Call		
1	0	0	0	0	7	11	
2	0	0	3	0	10	14	
3	0	0	0	0	1	11	
4	0	0	4	0	5	13	
5	0	1	5	0	6	13	
6	0	0	8	0	11	16	
7	NA	0	5	0	1	18	
8	6	3	0	0	10	6	
9	0	2	0	0	7	9	
10	0	0	7	0	4	11	
11	0	0	5	1	14	14	
12	0	0	0	0	3	5	
13	0	6	3	0	10	8	
14	0	0	2	0	2	4	
15	0	0	2	1	6	12	
16	0	3	8	0	12	15	



Table 5.3. Playback experiments with females of *Limnonectes palavanensis*. Number of female calls produced per playback test (N = 10).

Ind. #	Male Advertisement Call			Female Call		
	Male Advertisement Call	+	Male Courtship Call	+	Female Call	Female Call
		Female Call		Male Courtship Call		
1	2	6	6	8	4	
2	14	20	15	18	4	
3	20	8	10	14	6	
4	3	1	2	2	2	
5	1	2	1	2	1	
6	3	3	1	4	1	
7	3	6	5	5	2	
8	3	4	3	1	0	
9	6	13	13	10	6	
10	17	9	10	7	8	

## CONCLUSIONS

I studied the reproductive behavior of the smooth guardian frog *Limnonectes palavanensis*, using a combination of extensive fieldwork, bioacoustics, and methods in behavioral ecology and animal communication. *Limnonectes palavanensis* is one of the two species in the genus where the male guards a single clutch of eggs and transports the tadpoles to small bodies of water. I spent twelve months in the rainforests of Borneo making careful observations and carrying out field experiments describing previously unknown behaviors of this species. Males of *L. palavanensis* spent the majority of their time attending a single clutch of eggs until hatching and do not give advertisement calls while attending a clutch, suggesting that they are not trying to attract additional females. This suggests that males are forfeiting additional mating opportunities and thus can potentially become the limiting resource. Furthermore, tadpole transport behavior extends males' "time-out" from the breeding pool. Suitable deposition sites are rare, but the time males spend searching is unknown. Males' decisions about where to deposit his tadpoles seems to be influenced by the availability and permanency of bodies of water, rather than other factors such as the presence of a predator or conspecific tadpoles. Additionally, males exhibited a clutch partitioning behavior by depositing some of their tadpoles in separate pools, possibly to maximize their fitness in case of desiccation of the pools.

I described the complete vocal repertoire of both males and females of *L. palavanensis*. Males give an advertisement call to attract females and alert other males of their presence. Females give spontaneous courtship calls, and males produce a previously unknown courtship call which is only given as an immediate response to the female call. In *L. palavanensis*, females are highly vocal, exhibiting high calling rates compared with the exceptionally low calling rates

of the males. This behavior, where females outsignal males has not been previously observed in anurans. These behaviors observed in females of *L. palavanensis* coupled with observations of several females approaching a male provide evidence of a female-biased operational sex ratio, a characteristic of a sex-role reversed mating system.

During playback experiments, males appear to use acoustic interference as a form of male-male competition in simulated close-range interactions with another male in the presence of a female. Playback experiments also confirmed that the soft close range call produced by the males is a courtship call elicited by the female call alone. Playback experiments with females did not provide evidence of female-female competition; they also did not provide evidence that the female call functions as an aggressive call towards other females. Therefore, it seems that intrasexual competition among males is more intense than among females, despite the differences in calling rates between the sexes.

A case of true sex-role reversal in anurans has not been documented because no system has been found that satisfies all criteria for it. *Limnonectes palavanensis* is the strongest candidate to date. With its male parental care and female courtship displays, *L. palavanensis* offers a unique opportunity to test predictions of sexual selection theory and determine the key factors responsible for the evolution of sex-role reversal. In the different chapters of this dissertation, I have described aspects of the natural history and the vocal behavior of this species that were previously unknown. Together, these constitute solid evidence for a case of sex-role reversal. The predictions tested in this dissertation have broad theoretical implications for the fields of behavioral ecology and sexual selection. This research serves as the foundation for pursuing more in-depth questions about the evolution of sex-role reversed mating systems in this study system.