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Abstract. Amphibians exhibit diverse reproductive behaviors, including nine documented types of amplexus, the behavior in which male and female frogs position themselves for courtship, oviposition, and fertilization. All known forms of amplexus involve the male on top of or in line horizontally (cloacal apposition) with the female. Here, we report a novel form of amplexus observed in Lau's leaf litter toad (*Leptobrachella laui*; Megophryidae) in Hong Kong, China. Termed "sex-reversed inguinal amplexus," the female climbs on top of a male and the male transports the female to a concealed breeding site. We were unable to determine whether this was the amplectant position in which frogs engaged during oviposition or solely during courtship and prior to oviposition, but there are a number of possible evolutionary drivers that may have given rise to this behavior, including limiting suitable oviposition sites or strong competition for males among females. Further research will be necessary to understand the evolutionary origins of this novel reproductive behavior.

Key words: amphibian; amplexus; behavioral ecology; *Leptobrachella laui*; Megophryidae; oviposition.

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Introduction

Frogs display diverse reproductive behaviors (Wells 2007, Crump 2015, Pough et al. 2016), many arising through sexual selection. Higher species richness in the tropics has yielded an associated richness in courtship behaviors by both sexes that lead to pair formation, as well as exceedingly complex forms of parental care including egg attendance and hydration, and guarding or transporting larvae or provisioning them with food resources (Crump 1996, Haddad and Prado 2005, Pough et al. 2016). About 3000 species of frogs have been described in the past three decades (Crump 2015), and with those new species have come diverse and novel reproductive behaviors.

Besides courtship and parental care, diversity in reproductive behavior extends to amplexus. Latin for "embrace," amplexus refers to the behavior in which a male clasps a female before and during oviposition. Generally, amplexus allows the cloaca of the male to be positioned close to the female's cloaca, potentially increasing fertilization success and reducing the chance of another male fertilizing some of the eggs

(Duellman and Trueb 1986, Wells 2007). However, there are exceptions in some forms of amplexus, for example, dorsal straddle, in which the cloacas of the two sexes are not juxtaposed during oviposition (Willaert et al., 2016).

Diversity in amplexus has foundations in phylogeny and the environment, with axillary and inguinal amplexus being the two most common forms (Carvajal-Castro et al. 2020). Inguinal amplexus, in which the male grasps the female around the waist, is considered the ancestral condition. It has been secondarily derived in some species that burrow for oviposition; presumably, the alignment of the male farther back on the female's dorsum makes it easier for the female to move through leaf litter or soil as she pulls the male along behind her (Wells 2007). Axillary amplexus, in which the male clasps the female just behind her forelimbs, is a derived behavior (Duellman and Trueb 1986). There are a number of hypotheses concerning the mechanism driving this derivation. This position brings the cloacas of males and females closer together and may be beneficial in species that lay their entire complement of eggs as a single mass in a short time period (Wells 2007). Others have noted the relationship between groups with axillary amplexus and oviposition in lotic waters (Lynch 1973).

In the past few decades, other forms of positioning for egg deposition and fertilization have been discovered. To date, nine different forms of amplexus have been described for frogs, all involving the male on top of or in line horizontally (cloacal apposition) with the female, but not all involving clasping (Carvajal-Castro et al. 2020). For example, in cloacal apposition, the male and the female face in opposite directions with their cloacas either adpressed or in close proximity. The male either fertilizes eggs as the female releases them (external fertilization) or transfers sperm directly to the female's cloaca for internal fertilization. This form of amplexus occurs in frogs that oviposit in terrestrial habitats or are ovoviviparous (Vitt and Caldwell 2014).

Amplexus is considered to be highly conserved in anurans over evolutionary time (Wells 2007), as evidenced by the description of only nine forms of amplexus in the more than 7100 described anurans, a group that has roamed the earth for at least 260 million years (Anderson et al. 2008).

First described in 2014 (Sung et al. 2014), Lau's leaf litter toad (*Leptobrachella laui*) is a small toad with a limited distribution in Hong Kong and Guangdong Province, China, that breeds in small forested streams. Here, we report the discovery of a novel amplexus position involving the female on top, clasping the male, in Lau's leaf litter toad from Hong Kong. We refer to this new mating position as "sex-reversed inguinal amplexus." Given that we know little about the life histories of many recently described amphibian species, documentation of novel reproductive behaviors is important to increasing our understanding of the diversity and evolution of reproduction in frogs.

Methods

In 2010, we observed two instances of sex-reversed inguinal amplexus in Lau's leaf litter toad in the field. As the reproductive ecology of frogs in this genus was largely unknown and sex-reversed inguinal amplexus had not been previously documented in any frog, we undertook 30 surveys distributed between two streams known to harbor populations of this species to further document this behavior. Field surveys were conducted between 2010 and 2017 in Hong Kong. Each survey began approximately 30 min after sunset and consisted of one or two surveyors walking slowly within the stream and listening for calling males. Once a calling male was detected, surveyors sat quietly about 5 m away and watched the male using red LED lights to prevent altering the behavior of the frogs. We waited for up to 2 h with each male, depending on calling duration, in attempts to document interactions between males and females. One or two males were observed on a single survey, and surveys ended when calling became sporadic or ceased on a given night. We carried either a digital still camera or video camera to document observations.

In addition to field surveys, we brought males and females into the laboratory to videotape this behavior. Frogs were held in 38-L tanks, filled to a depth of about 5 cm with dechlorinated water, and equipped with a submerged water pump to simulate water flow conditions in a stream.

Stones were stacked up in the aquarium to provide crevices in which oviposition could occur. In March 2015, two males and one gravid female were captured and held for approximately 30 d. Video cameras were placed on the top of the tank and set to run from 18:00 to 6:00 every day. In February 2017, three males and two gravid females were held together in the tank for approximately 35 d. Video cameras were installed on top and on both sides of the tank and set to run as described above.

Field and laboratory observations

In the field, we observed three instances of sex-reversed inguinal amplexus in Lau's leaf litter toad. On 10 February 2010, we observed a pair of Lau's leaf litter toads (Megophyridae: *Leptobrachella laui*) engaged in amplexus along a stream within a private nature reserve in Hong Kong Special Administrative Region, China. We captured the frogs and noted that the individual on the top was female and the individual on the bottom was male, as opposed to the usual arrangement in which the male is on top. The female was identified based on the presence of eggs, which were visible through the abdominal skin.

On 5 May 2010, we observed the second instance of this behavior in Tai Mo Shan Country Park, Hong Kong. A male was calling to a female, which was sitting about 10 cm away on a rock in a stream. The female approached the calling male from behind and clasped the male around the waist. With the female on top, the male hopped toward a small rock crevice (approximately 5 mm above the water surface). During the journey to the crevice, the pair separated and re-engaged in amplexus three times, each time with the female on top. The amplectant pair eventually separated and the female followed the male into a rock crevice, where subsequent behaviors were unobservable. This observation lasted for about 15 min.

After the first two observations, we conducted 30 surveys and observed the behavior of 50 calling males. On 29 March 2017, we observed the behavior for a third time and photographed for the first time a pair of frogs in sex-reversed inguinal amplexus in Tai Mo Shan Country Park (Fig. 1). After the pair was photographed, the female released the male, and further observations could not be made. Attempts to videotape sex-reversed inguinal amplexus in the field were unsuccessful.

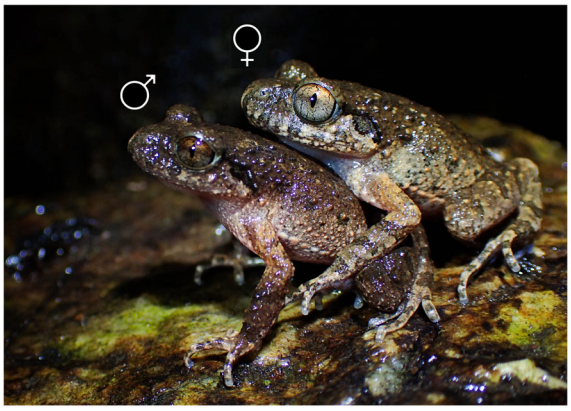

Fig. 1. A pair of Lau's leaf litter toads (*Leptobrachella laui*) in sex-reversed inguinal amplexus, in which a female is on top of a male, in Hong Kong, China in March 2017.

We videotaped the behavior five times in the laboratory, first on 6 April 2015 (Video S1). A clutch of eggs was found the following morning, but the behavior during oviposition could not be filmed because it occurred outside of the camera's view.

In 2017, we recorded four instances of sex-reversed inguinal amplexus on 22 February, 4 March, 18 March, and 23 March (Video S1). In each case in the video, the female appears slightly larger and exhibits a distended lateral abdominal region that is more whitish in color than that of the male. In the last instance, on 23 March, a single egg can be seen in the video protruding from and adhering to the female's cloaca. The following day, a clutch containing 67 unpigmented eggs was found. Oviposition was not captured on the video, however, because it occurred under rocks and out of the camera's view.

Discussion

In this study, we documented a novel amplexus position in anurans—sex-reversed inguinal amplexus. We were unable to determine the function of this novel reproductive behavior exhibited by Lau's leaf litter toad. This period of amplexus could be part of courtship (e.g., Carvajal-Castro et al. 2020), a signal by the female that

she will mate with the male, which is then followed by another form of amplexus during oviposition and fertilization. This is not an unlikely scenario, as other species of frogs engage in different forms of amplexus prior to and during oviposition (e.g., Pombal et al. 1994, Bourne et al. 2001, Willaert et al. 2016). Alternatively, it could be the actual mating position. Either way, discovery of this novel reproductive behavior raises a number of points to consider within the context of behavioral ecology and sexual selection. We can conceive of two possible evolutionary forces driving this behavior.

First, suitable oviposition sites may be limiting. If so, prior identification of a site by a male may confer reproductive efficiency and decrease risk of predation during mating. Despite searches by a number of herpetologists in the region over decades, eggs of this species have not been found. However, based on our observations and those of others, we believe that male Lau's leaf litter toads choose oviposition sites. Males have been reported to call outside of rock crevices (Karsen et al. 1998). We suspect that females oviposit in concealed rock crevices in these fast-flowing mountain streams, as do other small toads that breed in similar habitats. If suitable sites are limiting, males might identify oviposition sites and establish territories near those sites, from which they guard the sites and call to attract females. The males would then transport the females to those sites. Male selection of oviposition sites has arisen in phylogenetically and geographically distant taxa including in Bombay night frogs (Nyctibatrachidae: *Nyctibatrachus humayuni*) from India (Gramapurohit et al. 2011), fuscous foam frogs (Leptodactylidae, *Leptodactylus fuscus*; Wells 2007), Boraceia tree toads (Hylodidae, *Hylodes phyllodes*; Haddad and Sawaya 2000), and some poison dart frogs (Dendrobatidae; Crump 1972, Wells 2007) from Central and South America. The ability of a male to find and defend a suitable oviposition site and then attract a female to that site will be favored by sexual selection. Reproductive efficiency that reduces risk of predation is the most likely force driving selection for this behavior. Further support for this explanation may be found in the similar body sizes of males and females, in which females are only about 5% larger than males (Sung et al. 2014). In some other species of frogs that breed in fast-flowing streams and engage in ordinary amplexus, such as the harlequin frog (*Atelopus varius*), females may be up to 25% larger than males (Crump 1988), which allows them to more easily transport males for a protracted period of amplexus (Savage 2002) as the females search for suitable oviposition sites.

An alternative hypothesis is that strong competition for males among females may have driven selection for sex-reversed inguinal amplexus. If the number of males is limiting, sex-reversed inguinal amplexus may allow the female to secure her mate from other females until the pair retreats to an oviposition site. We believe that this is an unlikely explanation as, based on our observations, the sex ratio appears to be strongly male-skewed along streams during breeding seasons (Y. H. Sung, *unpublished data*), indicating that competition for males may not be intense among females. However, our observed sex ratio should be considered with caution as males are easier to detect because of their calls, and when not mating females may be active in the forest away from the stream, as in some South American stream-dwelling frogs (e.g., Lindquist et al. 2007), and therefore less likely to be detected around the stream. Determining the function and evolutionary mechanism that has driven development of this reproductive behavior will be challenging in a species with such cryptic reproductive habits.

It is not known whether sex-reversed inguinal amplexus occurs elsewhere in the lineage or has arisen in other unrelated species. Amplexus has not been observed in the species' closest relative the Fujian leaf litter toad (*Leptobrachella liui*). However, two newly described species, the orange-eyed litter frog (*Leptobrachella pyrrhops*) from Vietnam (Poyarkov et al. 2015) and the Tengchong leaf litter toad (*Leptobrachella tengchongensis*) from southwest China (Yang et al. 2016) exhibit ordinary inguinal amplexus, in which the male is on top of the female. The degree of sexual size dimorphism appears to be similar between these two species and that of Lau's leaf litter toad (Sung et al. 2014), suggesting that convergence in body sizes between males and females in Lau's leaf litter toad may not have resulted from release of constraints on this trait associated with ordinary amplexus.

The reproductive behavior of most *Leptobrachella* species has not been described. Importantly, we do not know whether this form of amplexus occurs during courtship only, with another type of amplexus used for oviposition, or whether sex-reversed inguinal amplexus extends through oviposition and fertilization. Further research will be necessary to understand the evolutionary force driving this novel reproductive behavior in Lau's leaf litter toad.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3407/full