Morphological changes in the female reproductive organs during mating in *Colostethus stepheni* and associated behaviour

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**Abstract.** Extended courtship and amplexus have been related either to readiness for spawning or to evaluation of the quality of egg-laying site. As preliminary observations revealed that a pair of *C. stepheni* can engage in two successive amplexi, an unusual pattern for dendrobatid frogs, we carried out experiments in the field to characterize this mating strategy. During the first hour after the first amplexus, we detect ovulation and changes in the oviduct morphology. The female could exhibit three different behavioural patterns, if disturbed after the first amplexus: remaining in the nest, leaving the nest and returning some minutes later, leaving the nest and subsequently enticed by their mate to another nearby nest. In cases where the male did not return for the second amplexus, the female sought another mate and engaged in a second amplexus.

**Introduction**

Dendrobatid frogs exhibit a great diversity of reproductive behaviours ranging from simple courtship and cephalic amplexus to prolonged and elaborated courtship without amplexus (Wells, 1977a; Duellman and Trueb, 1986; Zimmermann, 1990; Summers, 1992; Juncá, 1998; Pröhl & Hödl, 1999; Summers, 2000; Lima & Keller, 2003). With few exceptions they have semi-terrestrial tadpoles, i.e., in which embryonic development occurs in a terrestrial environment and tadpoles are carried by a parent to an aquatic environment, where the metamorphosis is completed (Duellman & Trueb, 1986; Pough et al., 1998). In general, males defend a territory, which includes appropriate calling sites and reproductive sites (Wells, 1977a, b).

*Colostethus stepheni* is a diurnal frog, like most of dendrobatids. The males defend a territory only during the reproductive period, which takes place during the rainy season. Nonetheless, their tadpoles are exceptional as the entire development is completely terrestrial, taking place on leaves within leaf litter (Juncá, 1998; Juncá et al., 1994), with or without accumulated water. Mating occurs in a nest that consists of a small chamber formed by leaves of the litter, where the clutch is deposited. The male guards the clutch especially in the first week of embryonic/larval development and parental care reduces predation (Juncá, 1996).

In addition to the complete terrestrial development of its tadpoles, *C. stepheni* has a reproductive mode unknown in other anurans: invariably, they engage in two amplexi of approximately 50 min each before ovipositing (Juncá, 1998). After having been attracted by a male and having selected it for mating, the female is lead to the nest where the first amplexus occurs. The male then leaves the nest while the female remains alone for a few hours. When the male returns, they engage in a second amplexus, followed by spawning. The clutch consists of approximately four eggs surrounded by dense jelly (Juncá, 1998). During the time interval between the two amplexi, the female’s abdomen expands noticeably. This suggests that the occurrence of two temporally separated amplexi is related to physiological changes in the female, involving ovulation and the production of the colloid...
substance that surrounds and protects the eggs. Alternately, the female could use the interval between the two amplexi to evaluate the egg-laying site. This behaviour has been observed (or suggested) for other anuran species having a long and complex courtship (Wells, 1977a; Kluge, 1981; Duellman and Trueb, 1986). For example, in *Hyla faber*, the female inspects the quality of the nest (Martins, 1988) during courtship. In *Epipedobates femoralis* and *E. trivittatus* the courtship lasts more than two hours (up to three hours in *E. femoralis*) before mating (Roithmair, 1994), when the male is choosing the nest site, and the female can reject the male by refusing to visit nest sites.

The aim of this study was to improve the knowledge of female premating behaviour in dendrobatid frogs by describing the female behaviour of *C. stepheni* during the reproductive process, including the changes that take place in the reproductive organs.

**Methods**

Field work was carried out in Reserva “41”, one of the reserves within the project “Dinâmica Biológica de Fragmentos Florestais” (DBFF) (Smithonians Institution/INPA), near Manaus, Amazonas, Brazil (2°25’S, 59°50’W, 110 m a.s.l.). More details concerning the study area and the reproductive biology of *C. stepheni* can be found in Lovejoy et al. (1984) and Juncá (1998), respectively.

Field work was carried out from January to March 1995. In order to obtain females of *C. stepheni* at different stages of the mating period, we built artificial nests and offered them to vocally active males. We built these artificial nests by joining together the concave sides of two fallen leaves (Juncá, 1998), thus forming an internal chamber (approximately 30 cm²) where mating could occur. We offered two artificial nests to each vocally active male (*n* = 200 males).

Daily observations of artificial nests were made between 6:00 to 10:00 h. During this period it was often possible to encounter a mating pair during its first amplexus. In order to investigate changes in the female reproductive organs during the five h period immediately following the first amplexus, 18 females were collected: three immediately after the first amplexus and three more at one h intervals during the five h after the first amplexus. Three additional females were collected at random to describe the reproductive organs of females that were not mating with mating females. Collected frogs were killed and preserved using usual techniques for anuran species (McDiarmid, 1994). These animals were later dissected to evaluate the morphology of the oviducts and the state of the oocytes in the ovaries or oviducts, employing a stereomicroscope equipped with a camera lucida. The females examined were deposited in the scientific collection at the Laboratório de Animais Peçonhentos e Herpetologia, Universidade Estadual de Feira de Santana (LAPH/UEFS) Bahia, Brazil, as numbers 222 to 236 and 1133 to 1138.

According to Loft (1974), we could observe three oviduct sections: anterior, median and terminal. In this study the characterization of each section was based on its distance from the ovarian funnel, thickness of the tissue, as well as the quantity of jelly secretions, mainly in the median section.

Thirty two other females were intentionally disturbed within the nests during different intervals after the first amplexus to observe their behaviour (1 h, *n* = 11; 3 h, *n* = 10; 5 h, *n* = 11). The intensity of the disturbances was standardized; each female was softly touched a maximum of three times with a dry leaf, and then three times with the observer’s finger. These touches were done quickly and separated by one or two seconds. For the statistical analysis, we used the Kruskal Wallis H test (Zar, 1984).

The behaviour of undisturbed females to the removal of the mating males before initiating the second amplexus was documented (*n* = 6).

**Results**

We observed 46 nests with male and female in first amplexus between 6:00 h to 8:00 h. After 8:00 h, in other 39 nests, we only observed the female after the first amplexus. All of these 39 nests contained eggs on the following day. We found clutches the day after inspection in only four nests without amplexing pair or female on the day before.

**The ovulation process**

All females had 2-6 mature pigmented oocytes in the ovaries or in oviducts (\(\bar{x} \pm SD = 4.0 \pm 0.89, n = 21\)). Mature oocytes in ovaries were 2.5-3.0 mm in diameter. Lighter pigmented oocytes (3-7) of approximately 1.0-1.5 mm in diameter and white oocytes of 0.3-0.7 mm in diameter were found only in the ovaries. The ovaries of unmated females (*n* = 3) and those females removed immediately after the first amplexus (*n* = 3) presented mature oocytes only in ovaries (table 1). However, the unmated females presented mature oocytes in different positions between other smaller pigmented oocytes. The
Morphological changes in the female reproductive organs

Table 1. Total number of mature oocytes in ovaries or in different sections of the ducts in females that did not mate \((n = 3)\) and at different time intervals after mating \((n = 3\) for each category). Number of females in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>Without mating</th>
<th>Immediately after mating</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
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</thead>
<tbody>
<tr>
<td><strong>Ovaries</strong></td>
<td>3-4 (3)</td>
<td>4-5 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (1*)</td>
</tr>
<tr>
<td><strong>Anterior oviducts</strong></td>
<td>4 (3)</td>
<td></td>
<td>4 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Median oviducts</strong></td>
<td>2 (1)</td>
<td>2-4 (3)</td>
<td>3-4 (2)</td>
<td>4 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Terminal oviducts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 (1)</td>
<td>4 (3)</td>
<td></td>
</tr>
</tbody>
</table>

* This female did not present mature oocytes in the oviducts.

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**Figure 1.** Representation of oviducts and oocytes during ovulation of *Colostethus stephensi* at four different times after the first amplexus. A: immediately after the first amplexus; B: two hours after the first amplexus; C: three hours after the first amplexus; D: five hours after the first amplexus. OF: ovarian funnel; OV: ovocyte; ARO anterior region of oviduct; MRO: medial region of the oviduct; TRO: terminal region of the oviduct; DT: digestive tube (beginning of esophagus, which was removed).

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females immediately after the first amplexus \((n = 3)\) had mature oocytes positioned in the most anterior portion of the ovaries. The oviducts were little developed and did not contain oocytes (fig. 1a). In one of the females collected immediately after the first amplexus, the median section of the oviduct walls yielded small gelatinous and transparent spheres when ruptured.

One hour after the first amplexus \((n = 3)\) the mature oocytes were in the anterior section of the oviducts. Only one of these females showed one oocyte in each oviduct in the median section. It was an amorphous white mass without oocytes in the other two females. Two hours after the first amplexus (fig. 1b), the females \((n = 3)\) showed the anterior and terminal sections of oviducts without oocytes and the median section with oocytes of 5.0 mm in diameter having a gelatinous capsule (based on their observed diameter). When the most external walls of the median region of the oviducts were ruptured, small transparent and gelatinous spheres were encountered. These spheres most certainly correspond to those eliminated together with the eggs during ovipositing. Three hours after the first amplexus one female showed oocytes only in the anterior section of oviducts. Two other females showed mature oocytes only in the median section, that appeared entangled and formed by a glandular tissue with the appearance of a dense white mass (fig. 1c). None of the three females had oocytes in the terminal section of oviducts. Two females removed four hours after the first amplexus had mature oocytes in the median section \((n = 2)\) and in the terminal section \((n = 1)\) of the oviducts. Finally, five hours after the first amplexus \((n = 3)\), the females had mature oocytes only in the terminal section of oviducts (fig. 1d). The oocytes were strongly pigmented and covered by a dense transparent gelatinous capsule. The oocyte capsule diameter was approximately 5 mm.
**Females disturbed after the first amplexus**

The females exhibited three different behavioural patterns after being disturbed between the first and second amplexus \( (n = 32) \):

- They remained in the nest. During some minutes after disturbance, some females \( (n = 11) \) flattened and extended their bodies over the substrate, did not move and remained within the nest. Females presented this behaviour in different periods after the first amplexus.
- They left the nest and returned there some minutes later. Seventeen jumped out of the nests but returned to it within 5 to 83 minutes. During their absence from the nest they remained approximately 30 cm from it. While returning, the females seemed to be cautious, and after pausing for a few seconds on top of the upper leaf of the nest, rapidly returned to it. The delays observed in returning to the nests varied according to the different times of disturbance: i) if disturbed 1 hour after the first amplexus, the females returned to the nest with a delay of approximately 50 minutes \( (\bar{x} \pm SD = 52.1 \pm 24.5 \text{ min}, n = 6) \); ii) if disturbed 3 hours after the first amplexus, the females returned to the nest after approximately 19 minutes \( (\bar{x} \pm SD = 19.1 \pm 12.2 \text{ min}, n = 7) \); iii) if disturbed 5 hours after the first amplexus, the females returned to the nest after approximately 19 minutes \( (\bar{x} \pm SD = 18.5 \pm 19.0 \text{ min}, n = 4) \). The differences observed were highly significant (Kruskal Wallis \( H = 7.16; df = 2; P < 0.05 \)).
- They left the nest and were subsequently enticed by their mate to another nearby nest. In 3 cases the male began to produce advertisement and encounter calls from the other artificial nest site to attract the female that was returning to the nest. In two of these cases, the female was disturbed 5 hours after the first amplexus. In both cases the pair initiated the second amplexus and oviposition occurred. In the third case, the female was disturbed one hour after the first amplexus. While she was returning to the nest, the male attracted her to a natural nest and attempted the second amplexus although it lasted more than 4 minutes, without oviposition. One female disturbed 5 hours after the first amplexus left the first nest and selected a neighbouring male for the second amplexus, possibly because the original male was captured and withheld by the observer. The neighbouring calling male attracted her, after she attempted several times to find the entrance to the first nest. Although it was an unusual time for vocalizations in this species \( (12:30 \text{ h}) \), a majority of the males were calling due to heavy rainfall. The neighbouring male courted and directed her to an artificial nest within his territory, where after a single amplexus \( (50 \text{ min}) \) she oviposited. All eggs were successfully fertilized.

**Males removed before the second amplexus**

Six males were removed just before returning to the nests. All females left these nests on the same day, between 14:39 h and 17:03 h, approximately three hours after male removal \( (\bar{x} \pm SD = 204.8 \pm 56.3 \text{ min}) \). In four cases the observer was able to follow the female until it encountered another calling male. These males courted and led the females to their nests. A single amplexus followed by oviposition was observed in all cases. All eggs were successfully fertilized.

**Discussion**

Anurans have a great variety of reproductive modes (see e.g. Duellman & Trueb, 1985). However, few studies with anuran species suggested how the courtship and amplexus are related with the timing of ovulation (Fortmann & Altig, 1973; Scarlata & Murphy, 2003) and to our knowledge none presented an examination of the female reproductive tracts during these events. Females of many species will not accept an amplexus until ovulation is complete or at least underway (Shalthe & Mechan, 1974). In other anuran species, however, the female will accept amplexus long before the time of oviposition, suggesting that the amplexus itself
Morphological changes in the female reproductive organs could play a role in bringing the female into a state of readiness for spawning (Salthe & Mechan, 1974). Wells (1977a) suggested that in some species in which male territories include individual oviposition sites with differences in quality, the females could choose males through the quality of their territory, and might delay ovulation until a favourable site is found. Our results agree with only the first suggestions. After the first amplexus, the female of *C. stepheni* ovulated, but if the female was disturbed in the nest, suggesting its low quality, she stayed in it or, if she left the nest site, she always tried to return to it.

Analyses of the female reproductive system of *C. stepheni* showed that ovulation occurs during the period between the first and the second amplexus. After the first hour, the mature oocytes have reached the median section of the oviduct, where they receive a gelatinous capsule. Females that were disturbed in the first hour after amplexus and left the nest remained outside for significantly more time before returning than did those females disturbed three to five hours after the first amplexus. Based on the changes in morphology of the median section of the oviducts, we conclude that the first hour after the first amplexus is a physiologically critical period for the female. During this period, it may be difficult for the female to return promptly to the nest. On the other hand, five hours after the first amplexus the oocytes reached the terminal section of the oviducts and the females might become physiologically more stimulated as the time of the second amplexus approaches. They would thus be more prone to return to, and stay in the nest where the second amplexus should occur. At least one study demonstrated that behavioural changes in female phonotactic preferences were related to different reproductive stages — prior to or immediately after mating and during ovulation (Lea et al., 2000).

A large majority of the females initiated ovulation in the morning. If males were removed before returning to the nest for the second amplexus, females moved towards another nearby vocalizing male. This behaviour occurs only at the end of the day or, exceptionally, after rain falls when males restarted their vocalizations. Neither the females that were disturbed in their nests, nor those whose males were removed before the second amplexus would interrupt the reproductive process already set in motion.

Although Bogart (1981) pointed out that there is little available evidence to define which is the derived or primitive reproductive mode in anurans, it has been considered that the aquatic development is the most primitive (Duellman and Trueb, 1986) and the semi-terrestrial tadpole with parental transport has been considered a synapomorph condition in the Dendrobatidae (Weygoldt, 1986). Based on our results, we suggest that the unusual behaviour of *Colostethus stepheni* to engage in two amplexi before oviposition might have arisen in response to physiological necessities imposed by changing from a semi-terrestrial reproductive mode to an entirely terrestrial one. Following Duellman and Trueb (1986) and Weygoldt (1986) statements, the terrestrial reproductive mode of *C. stepheni* is derived from the semi-terrestrial reproductive mode of other dendrobatids.

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**References**


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