A unique mating strategy without physical contact during fertilization in Bombay Night Frog (*Nyctibatrachus humayuni*) with the description of a new form of amplexus and female call

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Anurans show the highest diversity in reproductive modes of all vertebrate taxa, with a variety of associated breeding behaviours. One striking feature of anuran reproduction is amplexus. During this process, in which the male clasps the female, both individuals' cloacae are juxtaposed to ensure successful external fertilization. Several types of amplexus have evolved with the diversification of anurans, and secondary loss of amplexus has been reported in a few distantly related taxa. Within *Nyctibatrachus*, a genus endemic to the Western Ghats of India, normal axillary amplexus, a complete loss of amplexus, and intermediate forms of amplexus have all been suggested to occur, but many species remain unstudied. Here, we describe the reproductive behaviour of *N. humayuni*, including a new type of amplexus. The dorsal straddle, here defined as a loose form of contact in which the male sits on the dorsum of the female prior to oviposition but without clasping her, is previously unreported for anurans. When compared to known amplexus types, it most closely resembles the form of amplexus observed in Mantellinae. Furthermore, we prove that, opposed to the situation in most anurans, male semen release happens before egg deposition. We hypothesize that the male ejaculates on the female's dorsum and that sperm subsequently runs from her back and hind legs before fertilizing the eggs. A second feature characterizing anuran breeding is the advertisement call, mostly produced solely by males. Despite recent descriptions of several new *Nyctibatrachus* species, few studies have explored their vocal repertoire. We describe both the male advertisement call and a female call for *N. humayuni*. The presence of a female call has not been reported within Nyctibatrachidae, and has been reported in less than 0.5% of anuran species. Altogether, our results highlight a striking diversity and several unique aspects of *Nyctibatrachus* breeding behaviour.

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2 (*Nyctibatrachus humayuni*) with the description of a new form of amplexus and female call

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34 **ABSTRACT**

35 Anurans show the highest diversity in reproductive modes of all vertebrate taxa, with a variety of

36 associated breeding behaviours. One striking feature of anuran reproduction is amplexus. During

37 this process, in which the male clasps the female, both individuals' cloacae are juxtaposed to

38 ensure successful external fertilization. Several types of amplexus have evolved with the

39 diversification of anurans, and secondary loss of amplexus has been reported in a few distantly

40 related taxa. Within *Nyctibatrachus*, a genus endemic to the Western Ghats of India, normal

41 axillary amplexus, a complete loss of amplexus, and intermediate forms of amplexus have all

42 been suggested to occur, but many species remain unstudied. Here, we describe the reproductive

43 behaviour of *N. humayuni*, including a new type of amplexus. The dorsal straddle, here defined

44 as a loose form of contact in which the male sits on the dorsum of the female prior to oviposition

45 but without clasping her, is previously unreported for anurans. When compared to known

46 amplexus types, it most closely resembles the form of amplexus observed in Mantellinae.

47 Furthermore, we prove that, opposed to the situation in most anurans, male semen release

48 happens before egg deposition. We hypothesize that the male ejaculates on the female's dorsum

49 and that sperm subsequently runs from her back and hind legs before fertilizing the eggs. A

50 second feature characterizing anuran breeding is the advertisement call, mostly produced solely

51 by males. Despite recent descriptions of several new *Nyctibatrachus* species, few studies have

52 explored their vocal repertoire. We describe both the male advertisement call and a female call

53 for *N. humayuni*. The presence of a female call has not been reported within Nyctibatrachidae,

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56

57 Subjects Animal Behaviour, Zoology, Ecology

58 Keywords Amphibians, Advertisement call, Amplexus, Bioacoustics, Breeding behaviour, Egg

59 attendance, Egg predation, Nyctibatrachidae, Reproductive modes, Western Ghats

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INTRODUCTION

Anurans exhibit considerable diversity in their reproductive modes, with differences occurring, for example, in oviposition sites, larval development, and parental care (*Crump, 1974*; *Haddad & Prado, 2005*; *Crump, 2015*). One behaviour characteristic of nearly all anuran species' reproduction is amplexus. This behaviour, whereby the male takes the female in an embrace, is considered to have evolved to optimize successful fertilization of the eggs. Because fertilization is external in most anurans, the juxtaposition of the male and female cloacae during amplexus helps to synchronize egg deposition and the release of sperm (*Duellman & Trueb, 1986: 68–70*; *Wells, 2007: 452–458*). Several forms of amplexus have been described, with inguinal and axillary being the most widespread. Inguinal amplexus, in which the male clasps the female around her waist, is considered the ancestral state and is found in the oldest anuran lineages (*Duellman & Trueb, 1986: 68–70*; *Wells, 2007: 452*). Axillary amplexus, in which the male grasps the female in her armpits, is sometimes considered to be more efficient because the cloacae are better juxtaposed (*Rabb & Rabb, 1963*). While there is no evidence that axillary amplexus results in higher fertilization rates than inguinal amplexus, species with axillary amplexus may require less time to lay their eggs, thereby reducing an amplectant pair's vulnerability to predators (*Wells, 2007: 456*). Other forms of amplexus, in addition to axillary amplexus and inguinal amplexus, have evolved in some lineages (*Duellman & Trueb, 1986: 68–70*). A complete loss of amplexus has been observed in a few distantly related taxa (*Limerick, 1980*; *Kunte, 2004*; *Zhang et al., 2012*).

Another distinctive behaviour associated with anuran reproduction is calling. The vocal repertoire of male frogs and toads is well known, with the advertisement call, which is produced to attract mates and signal presence towards other males, being present in all but a few species (*Wells & Schwartz, 2007*). Male advertisement calls are species specific and can convey information about the signaller to other individuals. A key function of male advertisement calls is in premating species isolation (*Gerhardt & Huber, 2002*). It is, therefore, important to record and describe advertisement calls, as they can provide useful information in both taxonomical and evolutionary frameworks. Female calls are less well known because they are rare and seldom observed. For example, females in only a few anuran species are known to produce true advertisement calls (e.g., *Emerson, 1992; Bush, Dyson & Halliday, 1996; Bush, 1997; Bosch & Márquez, 2001*). The use of courtship calls by females engaged in close-range interactions with

93 males has been reported in a number of species (e.g., *Given, 1993*; *Tobias, Viswanathan &*
94 *Kelley, 1998*; *Bosch, 2002*; *Shen et al., 2008*; *Cui et al., 2010*). In some species, females produce
95 a territorial call upon disturbance (*Capranica, 1968*; *Wells, 1980*; *Stewart & Rand, 1991*).
96 Females in some anuran species also produce a release call when they are amplexed but
97 unreceptive, or amplexed by an undesired male (*Brzoska, Walkowiak & Schneider, 1977*;
98 *Gollmann, Benkö & Hödl, 2009*).

99 The genus *Nyctibatrachus Boulenger, 1882* is endemic to the Western Ghats of India and
100 comprises 28 known species, many of which have only recently been described (*Das & Kunte,*
101 *2005*; *Biju et al., 2011*; *Gururaja et al., 2014; Frost, 2015*). *Nyctibatrachus* species vary in
102 snout-vent length (SVL) from 10.0 mm (*N. minimus Biju et al., 2007*) to 76.9 mm (*N. grandis*
103 *Biju et al., 2011*) and are either stream-associated or found in the leaf litter, the latter making use
104 of small puddles for reproduction (*Biju et al., 2007*; *Biju, et al., 2011*; *Van Bocxlaer et al., 2012*).
105 Information about their natural history is scarce, and the conservation status of many species is
106 unknown, with only 15 out of the 28 species having been assessed by the IUCN Red List
107 (including four that are considered data deficient) (*Biju et al., 2011*; *IUCN, 2014*). Furthermore,
108 male advertisement calls have only been described in four species (*Kuramoto & Joshy,* 2001;
109 *Gururaja et al., 2014*).

110 Despite their poorly understood ecology, several studies suggest the presence of
111 interesting forms of reproductive behaviour (*Kunte, 2004*; *Biju et al., 2011*; *Gramapurohit,*
112 *Gosavi & Phuge, 2011*; *Gururaja et al., 2014*). In all *Nyctibatrachus* species, egg clutches are
113 deposited on rocks or vegetation overhanging water, and tadpoles fall in the water after hatching,
114 where they continue their development and metamorphosis (*Biju et al., 2011*; *Gururaja et al.,*
115 *2014*). Different kinds of amplexus behaviours have been described within this genus. Pairs of *N.*
116 *kumbara Gururaja et al., 2014*, for example, perform a short axillary amplexus followed by a
117 handstand. The female then deposits the eggs from this upside down position directly after the
118 male has dismounted (*Gururaja et al., 2014*). Afterwards, males of *N. kumbara* cover the
119 deposited eggs with a layer of mud, a behaviour previously unknown for anurans. In *N. aliciae*
120 *Inger et al., 1984*, *N. humayuni Bhaduri & Kripalani, 1955*, *N. jog Biju et al., 2011* and *N. minor*
121 *Inger et al., 1984* a short, loose physical contact between the male and female takes place as the
122 male sits on the dorsum of the female but does not clasp her as in a normal axillary amplexus
123 (*Biju et al., 2011*; *Gramapurohit, Gosavi & Phuge, 2011*). In other species, such as *N. petraeus*

124 *Das & Kunte, 2005*, amplexus behaviour may be completely absent, with the female depositing
125 the eggs prior to the male fertilizing them (*Kunte, 2004*; *Das & Kunte, 2005*). On one occasion,
126 amplexus was observed in *N. petraeus* five minutes before egg deposition took place, and the
127 author therefore considered this to be a type of 'pseudo-amplexus' (*Kunte, 2004*).

128   Together the available data indicates a wide range of breeding-associated behaviours in
129 *Nyctibatrachus*, with a new form of amplexus and an intrageneric variation in amplexus types
130 previously unreported in anurans. To better understand the reproductive behaviour of
131 *Nyctibatrachus* frogs, we observed breeding and associated vocalizations in a wild population of
132 *N. humayuni*. Here, we describe both the male advertisement call and a female call, and discuss
133 the different behavioural steps involved in reproduction based on field observations.

134

135 MATERIALS AND METHODS
136 Field surveys and behavioral observations

137 We spent a total of 40 nights in the field during July and August 2010 and 2012 studying a
138 population of *Nyctibatrachus humayuni*. The population was located in a dense forest near
139 Humbarli village, Koyna, Satara District, Maharashtra (coordinates 17°24'10.8" N,
140 73°44'13.2"E, 827 m asl) (Fig. S1A). Males were easily located by their calls while females,
141 which were harder to find, were more often encountered by chance. Sex was determined by the
142 presence of femoral glands in males and their absence in females (*Biju et al., 2011*). The moment
143 a female was observed approaching a male, we started filming the event using a camera with
144 infrared function (Sony HDR-XR 550VE). In most such instances, we used infrared light to
145 avoid disturbing the animals and affecting their behaviour. Movies were analysed with iMovie
146 8.0.6 (Apple Inc.) to determine the duration of different steps of reproduction. In order to test the
147 assumption that fertilization happens after the female has left the oviposition site (*Gramapurohit,*
148 *Gosavi & Phuge, 2011*), Ziploc® bags were placed around egg clutches directly after deposition
149 on five occasions to hinder male contact with the eggs. To determine the duration of embryo
150 development, deposited and fertilised clutches were monitored every evening until hatching
151 occurred.

152

153 Call recording

154   The advertisement calls of eight males and the calls of one female were recorded on a solid-state
155   digital recorder (Marantz PMD620, 44.1 KHz sampling rate, 16-bit resolution) using a
156   directional shotgun microphone (Sennheiser ME 66). A minimum of 20 calls were recorded per
157   individual. Microphones were handheld and positioned at a distance of approximately 75 cm
158   from the target animal. Sounds were monitored in real time using headphones (Sony MDR-
159   V500). At the end of each recording, the calling individual was captured and its SVL was
160   measured to the nearest 0.1 mm using digital callipers. A portable digital balance was used to
161   measure body mass to the nearest 0.01 g. These two measures of body size were used to compute
162   a measure of body condition (i.e., length independent mass) following *Baker* (1992). Condition
163   was estimated as the residuals from a regression of the cube root of mass on SVL divided by
164   SVL.We used these measures of SVL body mass and body condition to assess whether any call
165   properties were correlated with body size and condition. Recorded frogs were released at their
166   calling site immediately after obtaining body size measurements. To avoid recording the same
167   individual twice, we only recorded animals that were widely spaced (> 15 m). Since the study
168   area was large (ca. 250 m × 30 m) and males of this species are territorial, being found sitting
169   next to or on previously deposited egg clutches on subsequent nights, the chance of recording the
170   same individual multiple times was negligible (*Gramapurohit, Gosavi & Phuge, 2011*). As call
171   properties can vary with temperatures in anurans (*Gerhardt & Huber, 2002*), we recorded both
172   dry bulb and wet bulb air temperatures (±0.2°C) at the animal's calling site using a thermometer
173   (Jennson Delux).

174   This study was conducted with permissions and guidelines from the responsible
175   authorities in the State Forest Department of Maharashtra. Study permit: D-22
176   (8)/Research/4543/2012-13, dated 1-03-2012. This study did not sample animals for any captive
177   or laboratory studies. All observations were made in the wild. Recorded frogs were released back
178   at their calling site immediately after measuring the body size and body mass.

179

180   Call analysis
181   We used Raven Pro 1.4 (*Charif, Waack & Strickman, 2010*) to measure 32 acoustic properties
182   for each of the 160 advertisement calls recorded from the eight males. Raven's waveform display
183   was used to measure 21 temporal properties, while 11 spectral properties were measured using
184   the spectrogram slice view (1024pt. FFT, Hanning window). A description of the measured

185   properties is provided in Table S1A. Properties are analyzed after *Bee et al.* (2013a; 2013b).

186   Coefficients of variation (CV = standard deviation/mean) were computed to describe patterns of

187   both within-individual (CV_w) and between-individual (CV_b) variation in call parameters and are

188   expressed her as percentages (*Gerhardt, 1991*). Correlation of any of the acoustic parameters

189   with physical characteristics (SVL, body mass and body condition) and temperature was

190   explored by performing Spearman rank correlations using the software package Statistica v7.1

191   (Statsoft). Due to the low sample size, these correlation analyses should be considered

192   exploratory and are not intended to test any specific hypotheses. Five temporal and four spectral

193   properties were measured for the 20 calls recorded from one female (Table S1B). Since we only

194   recorded the call of a single female, descriptive statistics and correlations with other parameters

195   could not be carried out.

196

197   **RESULTS**

198   Reproductive behaviour

199   Breeding took place in and around a stream in which the water level heavily depended on the

200   weather conditions (Figs. S1B and S1C). Males were observed calling from different positions

201   along the stream. They were perched on rocks, vegetation and fallen trees, all bordering or

202   overhanging the water (Video S1). Their vertical position ranged from partially submerged to up

203   to four metres high on trees. Females were observed moving slowly between the territories of

204   different males. A schematic overview of the reproductive sequence is shown in Figures 1 and 2,

205   and videos of the different steps are provided as supplementary information.

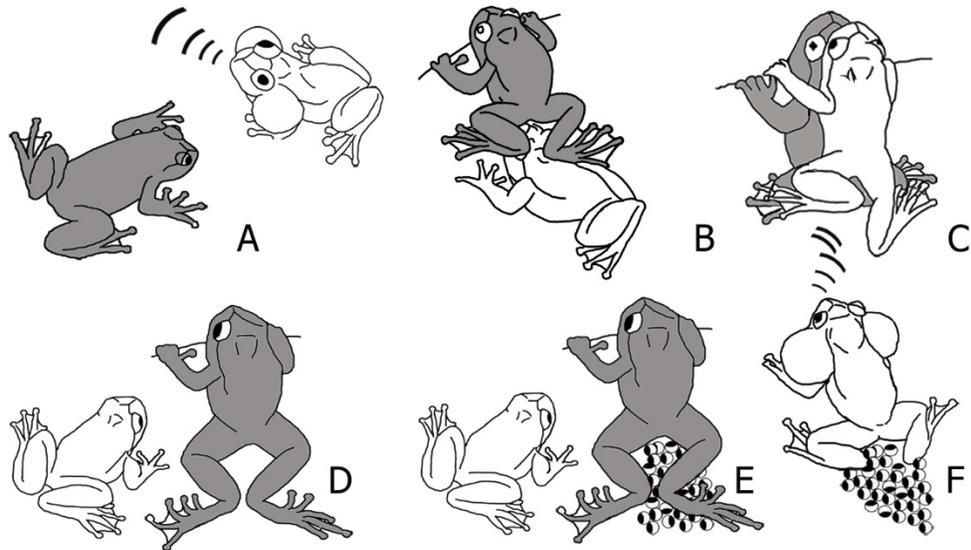



Figure 1 **Schematic sequence of reproductive behaviour in** *Nyctibatrachus humayuni.* (A) A female approaches a calling male. (B) The female sits in front of the male and creeps backwards, until her feet touch the male's head. (C) The male mounts the female and forms a dorsal straddle, and most likely sperm is released on her back during this moment. (D) When the female is about to deposit the eggs, the male dismounts. (E) Immediately after the male dismounts, the female deposits the eggs and remains motionless with her hind legs stretched around the eggs. (F) After oviposition, the female leaves the oviposition site, and the male sits on or near the eggs and continues to call. The male is drawn white and the female grey.

206   When a female approaches a male (Figs. 1A and 2A; Video S2), she sits in front of him
207   and creeps backwards until her abdomen is placed over his head, making physical contact (Figs.
208   1B and 2B; Video S3). At this moment, the male mounts the female. If the male does not react,
209   the female will repeat this physical contact. When mounted, the male rests on the female without
210   grabbing her in a firm amplexus, and instead uses his hands to hold on to the leaf or branch on
211   which the pair sits (Figs. 1C and 2C; Video S4). Lateral movements in the male's flank were
212   observed during this stage in a few of the breeding sequences (Video S4). This loose form of
213   amplexus is of short duration, lasting on average 768 s (n=21, range: 338–1670 s). At the end of
214   this embrace, the female strongly and repeatedly arches her back followed by the male
215   immediately dismounting (Fig. 1D; Video S5), a behaviour known from other species too
216   (*Gosner & Rosmann, 1959*). The female deposits eggs in a single bout immediately after the
217   male dismounts (Figs. 1E and 2D; Video S6). Oviposition takes only a few seconds and occurs

218   without any form of physical contact between the sexes. The female then remains motionless,
219   with her hind legs stretched around the freshly deposited clutch (Figs. 1E and 2D; Video S6). It
220   takes on average 479 s (n=17, range: 260–961 s) before the female moves after egg laying. After
221   this first movement, it takes another 728 s (n=11, range: 405–1692 s) before she jumps back into
222   the stream. During this period, the male is sitting nearby. There is, however, no physical contact
223   between the two sexes after egg deposition.
224

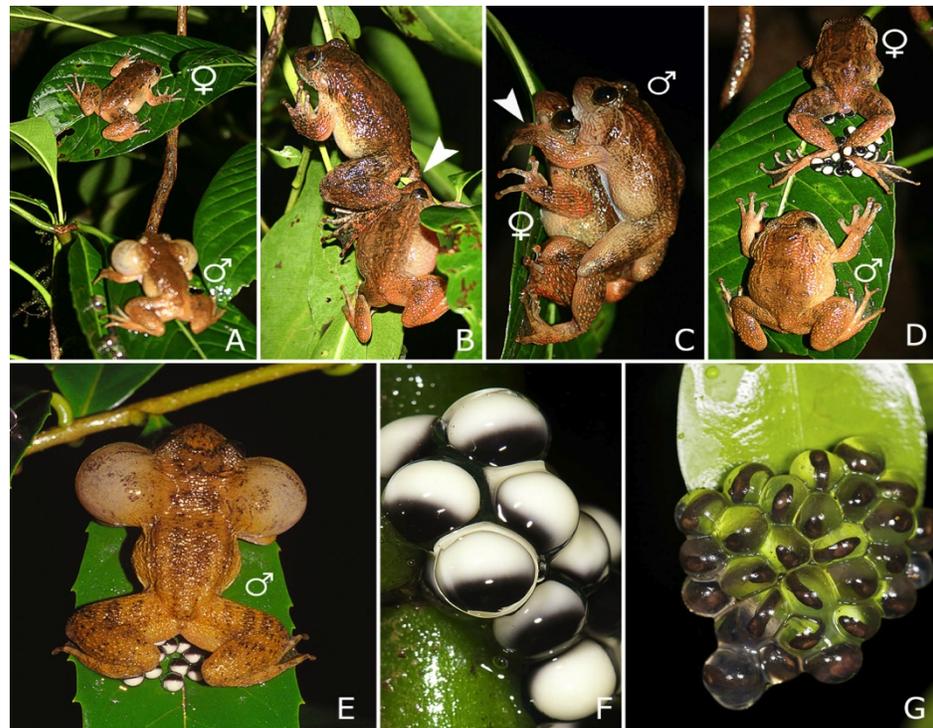



**Figure 2 A–E. Sequence of breeding behaviour in *Nyctibatrachus humayuni*;
F–G.** Egg development. (A) Female approaches a calling male. (B) Female
touches male just before the dorsal straddle (arrow indicates the position of
female's leg on male's head). (C) Male mounts the female in a dorsal straddle,
and most likely sperm is released on her back during this moment (arrow
indicates the male's hand positioned on the leaf, but not clasping the female). (D)
Female deposits eggs and remains motionless with her hind legs stretched around
the eggs. The male is mostly seen sitting close-by without any physical contact
with the female. (E) After the female leaves the oviposition site, the male sits on
or near the eggs and continues to call. (F) Freshly laid eggs, pigmented (egg
diameter 3.5 ± 0.2 mm, n=20). (G) Developing embryos on the 19th day, just

before hatching out of the eggs.

225   During several of the observed breeding sequences, the male crawled back over the eggs
226 after the female had moved away (Figs. 1F and 2E), but we did not see this on every occasion.
227 This prompted us to test whether fertilization had already happened during an earlier stage. We
228 tested this by placing a Ziploc® bag around five egg clutches directly after deposition, and in this
229 way prevented the male from having any further contact with the eggs (Video S7). Interestingly,
230 in all five clutches, the fertilization rate was 100%. In at least half of the observations we made
231 (nine observations), reproduction was interrupted by one or both of the frogs falling into the
232 water (Video S6, examples 6 and 7). Fallen individuals returned to the same position to continue
233 the mating sequence, except when strong currents swept them away. Males showed high site
234 fidelity, with new eggs being deposited directly next to present clutches or in close vicinity (≤ 50
235 cm). When a female approached a male calling from a position away from his previously
236 fertilized clutches, the male moved towards those eggs upon the first contact while continuing to
237 produce advertisement calls as the female followed. Males are known to defend their territories
238 (*Gramapurohit, Gosavi & Phuge, 2011*), and we witnessed a single event of aggression where
239 the resident male chased off an intruder (Video S8).

240   We monitored 15 egg clutches from the moment of deposition until hatching. Of the 15
241 clutches, 12 were eaten before hatching took place. These cases of predation were rarely
242 observed but could easily be distinguished from hatched clutches, because in hatched clutches,
243 the jelly remained visible on the substrate after hatching, in contrast to instances of predation,
244 where most of the jelly was removed. In one of the remaining three clutches, hatching started 18
245 days after oviposition and all larvae had fallen into the stream by the 19th day. In the other two
246 clutches, hatching started after 19 days and finished one and two days later (Figs. 2F and 2G).

247

248 Male advertisement calls

249 Male advertisement calls are composed of two distinct parts, with the first part being non-
250 pulsatile and the second part having a pulsatile temporal structure (Fig. 3A; Audios S1 and S2).
251 A detailed overview of analysed properties, including the variation both within and between
252 individuals, is given in Table S2.

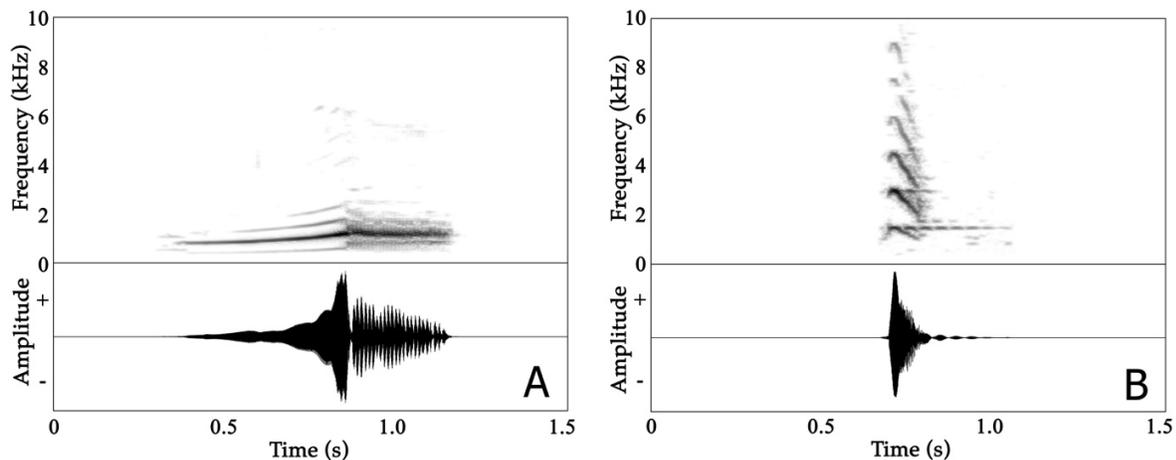
253   The mean call duration was 532 ms (range: 483–630 ms). On average, calls reached their

254   full amplitude in 268 ms (rise time) and decreased in amplitude over the last 262 ms of the call

255   (fall time). Both rise time and fall time were approximately 50% of the total call duration (Table

256   S2A). The second part of calls consisted of 16–24 pulses, with a mean pulse period of 13.2 ms

257   and a mean pulse duration of 12.8 ms. These pulses were produced at rates of 74–84 pulses/s.

258   Individual pulses had mean rise and fall times of 4.7 ms and 8.1 ms, respectively (Table S2C).

259

260   **Figure 3 Spectrograms (above) and oscillogram (below) of *Nyctibatrachus humayuni* calls.** (A) Male

261   advertisement call. (B) Female call.

262   The mean dominant frequency, measured over entire calls, was 1.34 kHz (1.11–1.45 kHz)

263   with relatively low variation between calls produced by the same individual (CV_w = 3.73%)

264   compared to the magnitude of variation observed among the eight individuals (CV_b = 8.19%).

265   The dominant frequency of the first, non-pulsatile part of the call ranged between 0.91–1.45 kHz

266   while in the second, pulsatile part the dominant frequency ranged between 1.17–1.47 kHz (as

267   calculated from first pulse, middle pulse and last pulse) (Table S2). Frequency modulation is

268   present in the first, non-pulsatile part of the call, but absent in the second, pulsatile part. The

269   dominant frequency increased by approximately 30% during the first part of the call, from 1027

270   Hz (mean dominant frequency 1) to 1336 Hz (mean dominant frequency 4) in less than 300 ms

271   (Table S2B).

272   Results of correlation tests are presented in Table S3. SVL and body mass of the different

273   recorded males and corresponding temperature data is presented in Table S4. The mean (± SD)

274   dry bulb and wet bulb air temperatures during our study were 22.2 ± 0.6°C and 22.7 ± 0.4°C,

275   respectively. There were no correlations between these temperatures and the acoustic properties

276   analysed. Though temporal properties are frequently correlated with temperature, we attribute

277   this lack of temperature effects on call properties to the very small temperature variation across
278   our recordings (< 2.1° C dry bulb air temperature; < 1.1° C wet bulb air temperature).
279   Several spectral properties were correlated with our measures of body size. The overall
280   dominant frequency of the entire call was significantly negatively correlated with body mass
281   (Table S3A). The dominant frequencies measured separately over the first and second halves of
282   the call were also significantly negatively correlated to body mass (Tables S3B and S3C). The
283   overall dominant frequency of the entire call was also negatively correlated with the SVL (Table
284   S3A). The overall dominant frequency, dominant frequency 2, and dominant frequency 4 of the
285   1st half were significantly negatively correlated with SVL (Table S3B). The overall dominant
286   frequency and dominant frequency of the first and last pulses of the second, pulsatile part of the
287   call were also significantly negatively correlated with SVL (Table S3C). The correlation of
288   dominant frequency 1 and dominant frequency 3 with SVL were marginally non-significant in
289   the first half of the call (Table S3B). Only one temporal property, pulse 50% rise time of
290   maximum amplitude pulse, was correlated with body mass (Table S3C).
291   With only one exception, there were no significant correlations between body condition
292   and the acoustic properties measured in this study (Table S3). The exception was the temporal
293   property of fall time of the first part of the call, which was significantly negatively correlated
294   with body condition (Table S3B). Several spectral properties had reasonably strong negative
295   correlations with body condition ($-0.69 \leq r \leq -0.62$), but these correlations were not quite
296   significant ($0.06 \leq P \leq 0.10$; Table S3B). Body condition can sometimes be strongly related to
297   certain temporal call properties associated with higher energetic demands, such as fast pulse rates
298   (*Jakob et al., 1996*). Although overall pulse rate was not related to body condition (Table S3C),
299   two measures of individual pulse period (= 1/pulse rate) had correlations with body mass that
300   approached significance ($r = -0.69$, $P = 0.06$; Table S3C). We attribute the general lack of
301   significant effects of body condition on several analysed call properties to our small sample size.
302
303   Female call
304   Female *Nyctibatrachus humayuni* calls were only rarely observed (four individuals), as females
305   do not seem to vocalize on a regular basis. A single female calling from a height of 2.5 m above
306   the ground was recorded. It produced about 50 calls in about 30 minutes. These calls sounded
307   similar to the calls we heard other females produce (Table S5; Video S9). The female call is

308   quite distinct from the male advertisement call (Fig. 3B; Audio S3; Video S9). The call is short,

309   consisting of a single note, with a mean call duration of 83 ms and a rapid onset (call rise time =

310   16 ms). The call's frequency spectrum is characterized by several frequency peaks, with the

311   lowest three peaks having mean frequencies of 1.45 kHz (Dominant frequency 1), 2.90 kHz

312   (Dominant frequency 2) and 4.37 kHz (Dominant frequency 3), respectively. The overall

313   dominant frequency of the call was 2.85 kHz (range 1.39–3.10 kHz). Based on measures of

314   within-individual CVs, spectral properties were less variable ($2.53 \leq CV_w \leq 12.37\%$) compared

315   to temporal properties ($18.30 \leq CV_w \leq 58.04\%$). The call 50% rise time and call 50% fall time

316   were more variable compared to other temporal properties. During two of our observations of

317   female calling behaviour in *N. humayuni*, the female only initiated calling when she had not

318   succeeded in reaching a calling male after trying for considerable time (more than 30 minutes).

319   On one of these occasions the male immediately changed his position as soon as the female

320   called. Although the male's response resulted in him calling from a new position closer to the

321   stream and to the female, she still failed to locate him.

322

323   **DISCUSSION**

324   New form of amplexus

325   The loose amplexus observed here in *Nyctibatrachus humayuni* differs from all previously

326   described amplexus types in anurans (*Duellman & Trueb, 1986: 68–70*) (Figs. 4A–F). Our

327   observations indicate there is some similarity between amplexus in *N. humayuni* and that

328   observed in some mantellid frogs, whereby the male sits with his abdomen on the female's head

329   (*Blommers-Schlösser, 1975*; *Glaw & Vences, 2007: 144, 186, 200, 204*; *Altig, 2008*). Duellman

330   & Trueb (1986: 69) defined this type of amplexus as a straddle, and it was later more specifically

331   called a head straddle (*Savage, 2002: 166*). The form of contact described in this study resembles

332   this head straddle, but the male is positioned lower on the female, with his abdomen placed on

333   her lower dorsum. When mounted, the male rests on the female without grabbing her tightly, but

334   instead uses his hands to hold on to a leaf, branch or tree trunk (Figs. 1C, 2C and 4J–K). On

335   some occasions his hands may rest on her arms or hands (Figs. 4H–I). Gramapurohit *et al.*

336   (2011) considered this to be cephalic amplexus, similar to that present in some dendrobatids.

337   Because the male does not press the backside of his hands against the female's throat, we believe

338   this term should not be used (*Wells, 1980*). We therefore propose to name this behaviour a *dorsal*

339   *straddle*. A dorsal straddle can be defined as a loose form of amplexus in which the male sits on

340   top of the female with his abdomen positioned on her lower dorsum. The male does not, however,

341   grasp the female under her armpits or head, but instead places his hands on the leaf, branch or

342   tree trunk the pair is sitting on (Figs. 1C and 2C). At the moment, dorsal straddle is known to

343   occur in *N. humayuni* only, but observations made in other *Nyctibatrachus* species (*Biju et al.,*

344   *2011*) might also correspond with this newly-defined type of amplexus.

345   We speculate that a loose form of contact, with the male holding on to the substrate rather

346   than to the female, as seen during a dorsal straddle, might function to avoid falling and

347   interrupting mating. In *Guibemantis depressiceps (Boulenger, 1882)*, which uses a head straddle

348   during amplexus, pulsing movements in the male flanks were observed during a short period of

349   physical contact (*Blommers-Schlösser, 1975*), similar to the ones observed here for

350   *Nyctibatrachus humayuni* (Video S4). The function of these pulsations is unclear, but similar

351   movements during amplexus have been observed in several other frog species (*Savage, 1932*;

352   *Rabb & Rabb, 1963*; *Weygoldt, 1976*; *Cui et al., 2010*). It has been proposed that these

353   movements might stimulate ovulation by the female (*Duellman & Trueb, 1986: 71*; *Cui et al.,*

354   *2010*).

355

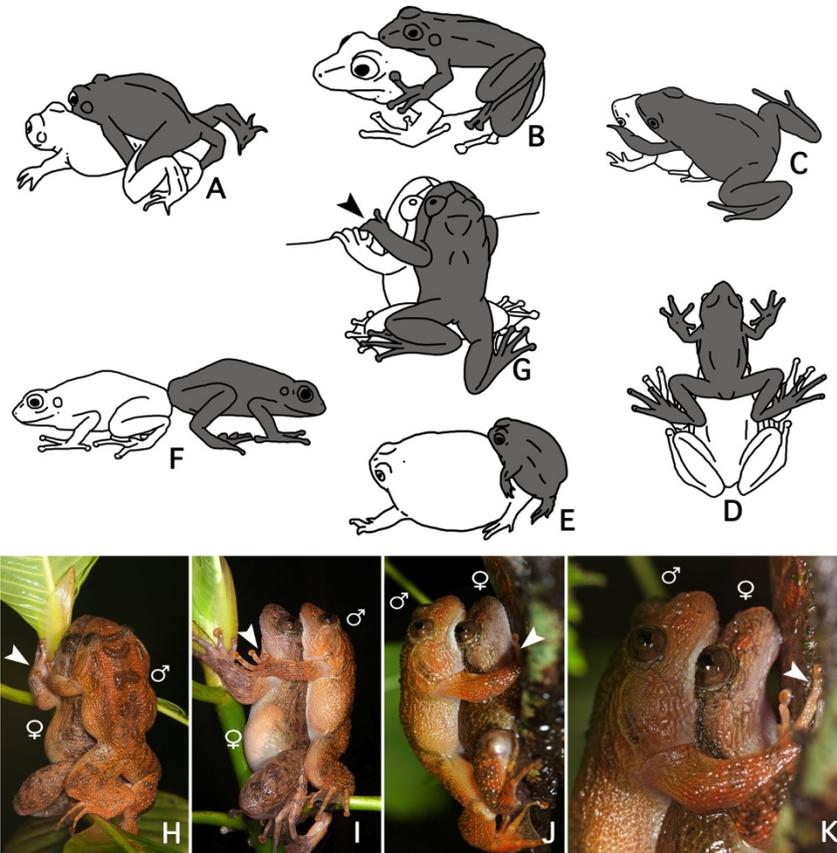



Figure 4 **A comparison of known amplexus positions found in anuran amphibians with the new amplexus mode in *Nyctibatrachus humayuni.*** (A–F) Known amplexus positions. (A) Inguinal. (B) Axillary. (C) Cephalic. (D) Head straddle. (E) Glued. (F) Independent (adapted from *Duellman & Trueb, 1986: 69*). (G–I) Dorsal straddle, with the male's hands on sides of the female's head but not clasping the female. (G–H) Dorsal views. (I) Side view. (J–K) Dorsal straddle, with the male's hands holding a twig but not clasping the female. (J) Side view. (K) Close-up of the side view. Arrows indicate the male grasping position. The male is drawn grey and the female white.

356

Moment of fertilization

358 The loose contact during amplexus, and the lack of any physical contact between both sexes
359 during oviposition, that we have described is rarely seen among anurans. Our observations
360 significantly extend those made by Gramapurohit *et al*. (2011) of the same species.
361 Gramapurohit *et al*. (2011) observed a subsequent step after oviposition, in which the male

362   placed himself on top of the eggs, and they considered this to be the moment of fertilization
363   (*Gramapurohit, Gosavi & Phuge, 2011*). In our study, however, all clutches covered with a
364   Ziploc® bag immediately after oviposition showed a 100% fertilization rate, proving that semen
365   release must happen before clutch deposition, since the male dismounts the female just before
366   oviposition (Videos S5 and S7). This is remarkable, since amplexus is considered to increase
367   chances of successful fertilization by synchronizing egg deposition and semen release (*Duellman*
368   *& Trueb, 1986: 68–70*). A complete lack of physical contact between male and female during
369   egg deposition is, therefore, rarely seen in anurans (*Wells, 2007: 458, 513*). Our findings are
370   inconsistent with the idea of the male fertilizing the eggs after deposition, as postulated by
371   *Gramapurohit et al.* (2011), and instead suggest that semen release must take place during the
372   dorsal straddle. We hypothesize that the male releases his sperm on the female's back and the
373   eggs are subsequently fertilized by the semen running down her back and hind legs. This
374   hypothesis is supported by the observation that female remains motionless after egg deposition,
375   for 479 seconds on average, with her hind legs stretched around the freshly deposited clutch
376   (Figs. 1E and 2D; Video S6). Furthermore, similar behaviour has been reported for *Blommersia*
377   *wittei* (*Guibé, 1974*), *B. blommersae* (*Guibé, 1975*), *Guibemantis depressiceps* and *G. liber*
378   (*Peracca, 1893*), among others (*Blommers-Schlösser, 1975*; *Glaw & Vences, 2007: 144, 186,*
379   *200, 204*; *Altig, 2008*). In these Malagasy frogs, males engage in a head straddle with females
380   and dismount the female before she finishes egg deposition.

381   In *Guibemantis depressiceps*, multiple males were observed sitting on a female's head
382   during a single egg deposition event, and the female exhibited lethargic behaviour, remaining
383   stretched around the clutch for at least an hour after deposition had finished (*Altig, 2008*). For
384   these mantellid frogs, the hypothesis of sperm running from the female back has also been
385   assumed (*Blommers-Schlösser,* 1975; *Altig, 2008*). The exact moment of semen release could not
386   be observed, as this was not possible with the naked eye during extremely wet field conditions, a
387   problem also mentioned by Altig (2008). The male might release his semen just before
388   dismounting the female. The eggs are then subsequently fertilized by sperm running down her
389   back. Another possibility is that sperm is gradually released during amplexus and that the eggs
390   are eventually deposited on top of the sperm. This last option has been observed in some species
391   of Dendrobatoidea (*Weygoldt, 1980*). A few anurans make use of internal fertilization (*Townsend*

392 *et al., 1981*; *Stephenson & Verrell, 2003*), but for *N. humayuni* this is very unlikely as males lack
393 an intromittent organ and only limited contact between the two sexes exists.

394   In *Nyctibatrachus petraeus* there is no physical contact at all, and it is believed that the
395 male fertilizes the eggs after the female has deposited the clutch (*Kunte, 2004*). In this species
396 the female is reported to immediately leave after egg deposition (*Kunte, 2004*), contrasting with
397 our findings of *N. humayuni* females. Gururaja *et al.* (*2014*) do not mention the moment of
398 fertilization in *N. kumbara*, but it is unlikely to happen as in one of the methods described above.
399 Since the female deposits the eggs from an upside-down position, sperm cannot run down from
400 her back onto the eggs. Furthermore, the male only touches the eggs with his hands after
401 deposition, to cover them with mud (*Gururaja et al., 2014*). Future studies regarding the
402 reproductive behaviour of *Nyctibatrachus* should aim to pinpoint when semen is released and
403 how fertilization is achieved.

404

405 Egg development and egg attendance
406 Of the egg clutches monitored, 80% were eaten by predators before hatching. During a single
407 occasion, a *Boiga* sp. (tree snake) was observed eating a *N. humayuni* egg clutch (Video S10).
408 Another water snake, *Rhabdops olivaceus (Beddome, 1863)*, was frequently observed around the
409 oviposition sites but no direct observation of predation was made. Gramapurohit *et al.* (*2011*)
410 reported an egg stage duration of 11 to 13 days in their study population, which is much shorter
411 than our observation of 18 to 21 days (Figs. 2F–G). An egg stage of 12 to 15 days and 8 days
412 were observed for *Nyctibatrachus petraeus* and *N. kumbara,* respectively (*Kunte, 2004*;
413 *Gururaja et al., 2014*), while for *N. aliciae* an egg stage of 8 to 9 days was observed in a clutch
414 studied in captivity (*Biju et al., 2011*). As egg development duration is negatively correlated
415 with temperature within a certain optimal range (*Duellman & Trueb, 1986: 120–124*; *Wells,*
416 *2007: 124, 499*), a higher number of observations and associated temperature data will be needed
417 to address this variation.

418   Males were seen each night in the same location, sitting near or on the developing
419 clutches that had resulted from earlier successful mating encounters. Egg attendance by the male,
420 or both male and female, is known for all *Nyctibatrachus* species in which reproductive
421 behaviour has been studied (*Kunte, 2004*; *Biju et al., 2011*; *Gramapurohit, Gosavi & Phuge,*
422 *2011*; *Gururaja et al., 2014*). The function of egg attendance has not yet been studied in this

423   genus, but it might prevent desiccation or reduce predation risk by arthropods, both of which
424   have already been confirmed in other anuran taxa (*Crump, 2015*). More specific parental care has
425   been documented for *N. kumbara* and *N. grandis*. *Nyctibatrachus kumbara* males cover the egg
426   clutch with mud, possibly preventing dehydration or providing camouflage against predation
427   (*Gururaja et al., 2014*). Males of *N. grandis* have been observed inflating their body upon
428   disturbance and making attempts to bite would-be predators, including human observers (*Biju et*
429   *al., 2011*).

430

431   Male call

432   The advertisement calls of only a few *Nyctibatrachus* species have been described (*Kuramoto &*
433   *Joshy, 2001*; *Gururaja et al., 2014*). Gururaja *et al.* (2014) provide a brief description of the calls
434   of *N. kumbara*, *N. jog* and *N. kempholeyensis (Rao, 1937)*, while a concise description of the call
435   of *N. major Boulenger, 1882* is given by Kuramoto & Joshy (2001). In *N. kumbara* and *N.*
436   *kempholeyensis* two distinct call types are distinguished; in *N. kumbara* one of these types was
437   produced more frequently when a female was present (*Gururaja et al., 2014*). Our recordings
438   were made in the absence of females. Therefore, we cannot exclude the possibility that in *N.*
439   *humayuni* too, distinct call types are present. The advertisement call from male *N. humayuni*
440   described here is complex, consisting of an initial, unpulsed part with frequency modulation and
441   a second, pulsed part without frequency modulation (Fig. 3A; Table S2). The call of *N. jog* also
442   has a pulsed second part of the call (*Gururaja et al., 2014*). The call of *N. humayuni* can be
443   distinguished from the call of *N. major*, the type I call of *N. kumbara*, and both types of call of *N.*
444   *kempholeyensis* by call duration. With an average duration of 0.53 s, the call is longer than the
445   type I call of *N. kumbara* (0.11 s) and the call of *N. major* (0.05 s), while being substantially
446   shorter than both call types of *N. kempholeyensis* (5.17 s and 11.69 s for the type I and type II
447   calls, respectively). Although call duration is similar between *N. humayuni* and the call of *N. jog*
448   and the type II call of *N. kumbara*, the dominant frequency of *N. humayuni* calls is lower (1.33
449   kHz) than that of *N. kumbara* type II calls (1.53 kHz) and *N. jog* calls (1.51 kHz).

450   Our results show a significant negative correlation between male body size and dominant
451   frequency (Table S3). This is the case for many anuran species, in which relatively larger
452   individuals' calls are characterized by relatively lower dominant frequencies. Larger males tend
453   to have more massive vocal cords and, consequently, produce lower-frequency calls (*Martin,*

454   *1971*). In some species, females show a preference for low frequency calls and hence larger
455   males (*Ryan, 1980*; *Wollerman, 1998*). However, experimental studies have also shown that in
456   other species no preference is given to lower frequencies (*Rosso, Castellano & Giacoma, 2006*).
457   Furthermore, female preference can vary between different populations within a single species
458   (*Schrode et al., 2012*). Within *Nyctibatrachus,* only a few species' calls have been described, and
459   no data on female preferences for spectral properties are available, making it impossible to draw
460   conclusions without further bioacoustic research on this group.

461

462   Female call

463   The discovery of a female call is remarkable, as this is rarely observed in frogs and toads.
464   Female calling behaviour has so far been reported in – to our knowledge – only 25 anurans,
465   representing less than 0.5% of the total of 6583 anuran species that are currently recognized
466   (25/01/2016) (*Frost, 2015*). An overview of the species for which a female call has been reported
467   is given in Table S6. Female calling in *Nyctibatrachus humayuni* was observed only briefly and
468   on just four occasions over a total of 40 nights in the field, compared to the almost permanent
469   presence of male advertisement calls. The female call of *N. humayuni* is shorter and less complex
470   than that of the male, consisting of a single, unpulsed note (Fig. 3B; Video S9). A short and less
471   intense female call is also observed in other anuran species (*Given, 1987*; *Emerson, 1992*; *Cui et*
472   *al., 2010*) and can, in part, be explained by the smaller size of laryngeal and oblique muscles of
473   the female (*Emerson & Boyd, 1999*). A female call is not known from any other species of
474   *Nyctibatrachus*. Since documentation of the presence of female calls can be done with more
475   confidence than their absence (*Wells, 2007: 282*), female vocalizations might be more common
476   than currently believed, both among *Nyctibatrachus* species and across all anuran taxa more
477   generally (*Emerson & Boyd, 1999*).

478   Observed male responses to female calls include positive phonotaxis and changes in male
479   vocalization rate (*Emerson & Boyd, 1999*; *Shen et al., 2008*; *Cui et al., 2010*; *Wang et al., 2010*),
480   suggesting that female vocalization is important in mate location and recognition. In this and
481   other *Nyctibatrachus* species, calling males are often located in difficult to reach locations (e.g.
482   vegetation overhanging the water) that might be accessed only by leaping towards them directly
483   from the stream. It is likely that in such a situation the female will mainly rely on acoustic cues
484   to localize the calling male. Intensified male calling, or the male shifting to another position, in

485   response to a female call might then prove beneficial in successfully locating a mate. This

486   hypothesis is only speculative at present, and additional observational and experimental studies

487   will be required to test it. Several other potential functions have been attributed to female calling

488   behaviour, such as signalling receptivity (*Tobias, Viswanathan & Kelley, 1998*; *Shen et al.,*

489   *2008*), distinguishing satellite males from territorial ones (*Given, 1993*) and inciting male-male

490   (*Judge, Swanson & Brooks, 2000*) and female-female competition (*Bush, 1997*; *Bosch, 2002*).

491   Few of these hypotheses, however, have been tested experimentally (*Wells & Schwartz, 2007*).

492   Both positive and negative phonotaxis has been observed in male *Xenopus laevis* (*Daudin, 1802*)

493   in response to two distinct female call types (*Wang et al., 2010*). Distinct female call types have

494   also been described for *Alytes cisternasii* (*Márquez & Verrell, 1991*). Together, the scarce data

495   available on female vocal behaviour and the variability observed in male responses suggest a

496   complex of multiple, non-exclusive functions rather than a sole explanation.

497

498   Diversity in reproductive behaviours

499   Our results, combined with other recent behavioural studies (*Kunte, 2004*; *Biju et al., 2011*;

500   *Gururaja et al., 2014*), show a striking interspecific diversity in reproductive behaviours within

501   *Nyctibatrachus*. As the reproduction of many species in this genus has not yet been studied, the

502   extent of variation is likely to be even greater. Our limited understanding of this diversity is

503   evidenced by the high number of recent publications reporting new modes of breeding and

504   associated reproductive behaviours (*Zhang et al., 2012*; *Gururaja et al., 2014*; *Iskandar, Evans*

505   *& McGuire, 2014*; *Crump, 2015*; *Seshadri, Gururaja & Bickford, 2015*; *Senevirathne et al.,*

506   *2016*). Within *Nyctibatrachus*, variation in the types of amplexus is especially remarkable,

507   ranging from a normal axillary amplexus to a complete lack of physical contact between the

508   sexes (*Kunte, 2004*; *Gururaja et al., 2014*). The use of amplexus is the ancestral state in anuran

509   amphibians and enables juxtaposition of male and female cloaca to ensure successful fertilization

510   (*Duellman & Trueb, 1986: 68–70*; *Wells, 2007: 452–458*). Secondary loss of amplexus was

511   already shown for a limited number of anuran taxa (*Limerick, 1980*; *Brown et al., 2008*; *Zhang et*

512   *al., 2012*) and a short, loose contact (head straddle) similar to the dorsal straddle reported here

513   for *N. humayuni,* was also found in several *Mantidactylus* species (*Blommers-Schlösser, 1975*;

514   *Altig, 2008*). Many males of both *Mantidactylus* and *Nyctibatrachus* possess distinct femoral

515   glands (Fig. S2B) (*Vences et al., 2007*; *Biju et al., 2011*). The function of these structures in

516 *Nyctibatrachus* has not yet been addressed, but recently it was shown that the femoral glands of

517 *Mantidactylus* secrete volatile pheromones (*Poth et al., 2012*; *Hötling et al., 2014*). Further

518 studies are required to discover and understand the full extent of variation and the evolutionary

519 advantage of these highly diversified behaviours within *Nyctibatrachus*, especially regarding the

520 modification and loss of amplexus.

521

522 **CONCLUSION**

523 The breeding behaviour of *Nyctibatrachus humayuni* has several unique elements: a new type of

524 amplexus, the release of semen before oviposition and the presence of a female call. These

525 findings further highlight the tremendous variation present in the reproductive behaviour of

526 anuran amphibians. *Nyctibatrachus* frogs are one of several unique taxa in the Western Ghats

527 biodiversity hotspot, which is heavily threatened by anthropogenic activities (*Myers et al., 2000*;

528 *Bossuyt et al., 2004*; *Van Bocxlaer et al., 2012*). A good understanding of each species' ecology,

529 including reproduction, is of major importance for planning and successfully implementing

530 conservation strategies. Additional studies further exploring the unique and diverse behaviour in

531 *Nyctibatrachus* frogs are, therefore, badly needed. Special attention should be paid to describing

532 the amplexus type, determining the moment of fertilization and assessing the presence and

533 function of female calling behaviour.

534

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542

543 **Additional Information and Declarations**

544

545 **Data Deposition**

546 The following information was supplied regarding data availability: No additional raw data

547 beyond the Supplemental Information found in the body of the manuscript.

548
549 **Supplemental Information**
550
551 Videos
552 **Video S1** Male advertisement call.mov (20:00 sec)
553 **Video S2** Female approaches calling male.mov (50:00 sec)
554 **Video S3** Female touches male's head with her feet.mov (24:00 sec)
555 **Video S4** Dorsal straddle: a new amplexus mode. mov (52:00 sec)
556 **Video S5** Female's movements and dismounting of male.mov (56:00 sec)
557 **Video S6** Ovipostion: female deposits eggs.mov (01:10 sec)
558 **Video S7** Field experiment: Bagging of eggs.mov (10 sec)
559 **Video S8** Male-male combat.mov (37 sec)
560 **Video S9** Female call.mov (27 sec)
561 **Video S10** Egg predation by snakes.mov (01:14 sec)
562
563
564 Audios
565 **Audio S1** Male advertisement call: 10 filtered calls. wav
566 **Audio S2** Male advertisement call: 10 unfiltered calls. wav
567 **Audio S3** Female advertisement call: 6 unfiltered calls. wav
568
569 Figures
570 **Figure S1** Study site and breeding habitat of *Nyctibatrachus humayuni*.
571 **Figure S2** An adult male of *Nyctibatrachus humayuni* (in life).
572
573 Tables
574 **Table S1** Description of the different properties that are used to describe the male and female calls.
575 **Table S2** Overview of descriptive statistics of male *Nyctibatrachus humayuni* advertisement calls, based
576 on the values determined from a sample of eight individuals.
577 **Table S3** Spearman Rank correlation between mean properties of male *Nyctibatrachus*
578 *humayuni* calls and SVL, mass and temperature (wet and dry) (n=8).
579 **Table S4** Snout-Vent Length (SVL) and body mass of recorded male individuals, with
580 corresponding dry bulb and wet bulb temperature.
581 **Table S5** Descriptive statistics of entire call of female *Nyctibatrachus humayuni* based on the
582 values determined from a sample of 1 individual (20 calls).
583 **Table S6** Overview of anuran species in which a female call has been described.
584
585
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