

**TREE HOLES TO TRASH: UNIQUE UPSIDE-DOWN
TERRESTRIAL SPAWNING, AGONISTIC INTERACTIONS,
COMPLEX MATING CALLS, AND UNNATURAL
BREEDING ALTERATIONS IN MINERVARYA
CHARLESDARWINI (ANURA, DICROGLOSSIDAE)**

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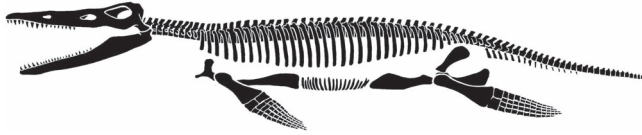
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TREE HOLES TO TRASH: UNIQUE UPSIDE-DOWN TERRESTRIAL SPAWNING, AGONISTIC INTERACTIONS, COMPLEX MATING CALLS, AND UNNATURAL BREEDING ALTERATIONS IN *MINERVARYA CHARLESDARWINI* (ANURA, DICROGLOSSIDAE)

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ABSTRACT. Anuran amphibians exhibit the greatest diversity of reproductive modes among tetrapod vertebrates. The Andamanese Charles Darwin's frog, *Minervarya charlesdarwini*, is the only species of the family Dicroglossidae that is known to naturally deposit eggs in water-filled cavities of tree holes or buttresses, where they then undergo exotrophic development. We describe the reproductive behavior in this species that involves a unique combination of traits: (1) Males produce complex advertisement calls comprising at least three different call types, in addition to a type of aggressive call. (2) Unpaired males exhibit agonistic interactions with each other and with mated pairs. (3) Mate selection, amplexus, and oviposition take place inside water-filled cavities. (4) During axillary amplexus, mating pairs synchronously switch between head-up and head-down positions above and below the water surface using both forward and backward movements. (5) At the time of egg laying, amplexant pairs are in an upside-down position on the cavity walls with their bodies completely outside the water. (6) Eggs are deposited over multiple bouts on the inner walls of the cavities and terrestrially above the water surface. Upside-down spawning in *M. charlesdarwini* is a unique trait among phytotelm-breeding terrestrial frogs. The combination of terrestrial oviposition sites in water-filled phytotelmata and the upside-down egg-laying posture is a novel report for the family Dicroglossidae and perhaps all anurans. This specialized behavior is also likely derived for a species that is embedded in a group of largely aquatic-breeding minervaryan frogs. Although *M. charlesdarwini* appears to be an obligate phytotelm breeder, individuals were often observed breeding inside cylindrical, water-filled plastic sapling bags in

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plant nurseries adjacent to fragmented forest patches, or in rain-filled discarded plastic, glass, or metal containers left as trash at the forest edge. Use of such unnatural breeding sites is likely a forced behavioral shift in response to rapidly changing forest landscapes associated with recent habitat loss and fragmentation. Our findings call for conservation attention to this habitat specialist, which, although locally abundant, is an endemic and threatened species of the Andaman Islands.

KEY WORDS: Amphibia; Andaman and Nicobar Islands; frog behavior; amplexus; bioacoustics; conservation; oviposition; phytotelm breeding; reproductive mode

INTRODUCTION

Among tetrapod vertebrates, amphibians exhibit the greatest diversity of reproductive modes (Duellman and Trueb, 1986; Wells, 2007). Depending on which set of characters is used to classify them, at least 41 and as many as 74 reproductive modes are documented (Haddad and Prado, 2005; Crump, 2015; Nunes-de-Almeida et al., 2021). Among anuran amphibians, deposition of eggs in aquatic habitats is generally regarded as ancestral and is observed in most phylogenetically basal groups (barring a few exceptions like the primitive group *Leiopelmatidae*), whereas terrestrial oviposition is considered a derived and far more specialized behavior associated with adaptive radiations that exploit terrestrial environments (Duellman and Trueb, 1986; Wells, 2007; Gomez-Mestre et al., 2012). As knowledge of the astounding diversity of reproductive modes across anuran phylogeny has accumulated, questions have emerged regarding the evolution of specialized modes (e.g., Gomez-Mestre et al., 2012).

The preferred microhabitat for oviposition is a key factor for species survival; it determines the quality of an offspring's environment and larval performance (Resetarits and Wilbur, 1989) and it directly affects their reproductive success (Resetarits, 1996; Abbott and Dukas, 2016). Factors that determine the selection of oviposition site in anurans include the presence of predators, desiccation risk, and the laying substrate (Resetarits and Wilbur, 1989; Egan and Paton, 2004; Vredenburg, 2004). Because of the complex life history of many frogs,

oviposition sites need to accommodate external fertilization, embryonic development, hatching, and subsequent larval development (Browne et al., 2015; Bowcock et al., 2009). A 2004 review identified 102 species of anurans worldwide that utilize rainwater stored in some parts of plants for breeding and are therefore classified as phytotelm breeders (Lehtinen, 2004), whereas a more recent compilation lists about 272 species in this category (Lehtinen, 2022). Collectively, these species represent only 13 of 58 currently recognized anuran families, the largest number being in *Hylidae* (68 species), *Dendrobatidae* (64 species), *Microhylidae* (53 species), *Bufo* (28 species), and *Rhacophoridae* (23 species). Among phytotelm breeders, only about 33 species from several families are known to deposit nonfroth (viz., not in a foam or bubble nest), nonaquatic (laid outside water) eggs on terrestrial substrates in water-filled reservoirs such as tree holes, nut husks, leaf axils, tree buttresses, and similar tree parts (i.e., modes 25 and 26 as per Nunes-de-Almeida et al. 2021). This behavior broadly corresponds to reproductive mode 26 of Haddad and Prado (2005) and Crump (2015). In species studied to date, the volume of water in phytotelmata ranges from several liters in large cavities (Schiesari et al., 2003) to extremely small quantities of just a few milliliters in small ones (Rödel et al., 2004). Although the list of phytotelm breeders that also use phytotelmata as oviposition sites remains far from complete (Nunes-de-Almeida et al., 2021; Biju et al., 2016; Garg et al., 2018), present knowledge

highlights the highly specialized nature of this reproductive mode.

The family Dicroglossidae Gray, 1825 is a large radiation of Asian frogs that comprises 15 genera and 220 species (AmphibiaWeb, 2024; Frost, 2024). The dicroglossid genus *Minervarya* contains 31 species primarily distributed in South and Southeast Asia, with major centers of diversity in India, Myanmar, Nepal, Bangladesh, and Thailand (Garg and Biju, 2021). The Charles Darwin's frog *Minervarya charlesdarwini* is an endemic but locally abundant species on the forest floor on numerous islands of the Andaman Archipelago (Garg et al., 2022). The breeding behavior of this frog is largely unknown, except for a cursory report of its phytotelmonous (phytotelm-inhabiting) tadpole that accompanied the original species description (Das, 1998) and a subsequent redescription of the tadpole with comments on breeding behavior (Gopika et al., 2023). *Minervarya charlesdarwini* is the only dicroglossid species known to deposit eggs in water-filled cavities of tree holes or buttresses (Gopika et al., 2023). We studied its breeding sequence and here report numerous novel features of its reproductive behavior, including an upside-down terrestrial spawning behavior that is unique among phytotelm-breeding frogs, male agonistic interactions, a complex vocal repertoire, and the alarming use of human trash as unnatural oviposition sites.

MATERIALS AND METHODS

Field surveys and behavioral observations

The study was conducted at two field sites on South Andaman Island, Andaman and Nicobar Archipelago, India: Mount Manipur National Park (MMNP), 11°43'12"–11°45'36"N, 92°43'48"–92°44'24"E, elevation 80–400 m; and Naya Sahar Botanical Garden (NSBG), 11°37'48"–11°38'24"N, 92°43'48"–92°44'24"E, elevation 10–20 m. The study site at Mount Manipur is

an evergreen forest, whereas the site at Naya Sahar village is an experimental garden-cum-arboretum and a regional center of the Botanical Survey of India, with naturally growing native trees along with cultivated plant species adjacent to a fragmented primary forest patch. During our studies at these locations in 2019, we observed water-filled tree holes with tadpoles and calling males of *M. charlesdarwini*. Dedicated studies were subsequently carried out over the next two field seasons (June–August 2020 and 2021) to study the reproductive behavior in this species. We spent a total of 55 nights (37 at NSBG, 18 at MMNP) studying the two populations. Eight oviposition sites were identified (six at NSBG and adjoining forest, two at MMNP) on the basis of the presence of the highest number of calling males and deposited eggs during the initial phase of the breeding season. Sex was determined by the presence of an external vocal sac in males and its absence in females. Behavioral observations such as calling activity, male–male combat, unpaired male combat with an amplexant pair, and amplexus and oviposition were videographed and photographed. Observations were made at night using headlamps with red or occasionally white light, which seemed not to disturb the mating pairs. Most breeding events discussed below are based on video recordings that were used to determine the sequence and duration of different events and behaviors, in addition to our direct field observations.

Call recording and call analysis

Calls from three male individuals of *M. charlesdarwini* were recorded at the NSBG and MMNP sites in June 2022 between 18:00 h and 02:00 h. Recordings were made on a hand-held Marantz PMD620 solid-state digital sound recorder at a sampling rate of 44.1 kHz with 16-bit resolution, which was attached to a Sennheiser K6 unidirectional microphone held 50–100 cm in front of the calling frogs. Before each recording, the input level of the recorder

was adjusted to prevent clipping of the amplitude envelope of the calls. This maintained a steady signal-to-noise ratio within the recordings. Dry bulb temperature (23–27°C) and wet bulb temperature (24–26.5°C) were recorded at the calling site.

Fourteen acoustic properties (13 temporal and one spectral) of each call were measured following the terminology of Bee et al. (2013) using Raven Pro 1.6.4 software (Charif et al., 2010). Oscillograms showing the amplitude-vs.-time waveform were prepared for visual representation of the calls. The overall dominant frequency was obtained using Raven's spectrogram function (1,024-point fast Fourier transform, Hann window, 50% overlap, 43.1-Hz resolution); spectrograms for the calls were prepared to match the time frame of the oscillograms.

RESULTS

Vocalizations

We identified four types of calls produced by male *M. charlesdarwinii* that were labeled following the methodology of Garg et al. (2021). Three of these were the most frequently produced call types, were produced in bouts, and collectively represent the male advertisement call repertoire. An additional call type was recorded and analyzed from two individuals engaged in an agonistic interaction and is considered an aggressive call.

Male advertisement calls (Figs. 1, 2; Tables 1, 2; Video S1; Audios S1–S3). We observed males of *M. charlesdarwinii* actively calling at both natural (water-filled tree holes, cavities, and buttresses) and unnatural (plastic, glass, and metal containers) sites. At natural sites, calling heights ranged from the forest floor to approximately 1.3 m high in trees, whereas calling individuals perched from ground level to 0.4 m heights at unnatural sites in water-filled objects. The inflated vocal sac was single, subgular, and grayish white. Males started calling from the ground or leaves on

low vegetation by 18:00 h and gradually moved closer to the water-filled cavities (Fig. 1). Advertisement calls typically were produced in short bouts or call groups that lasted from tens of seconds to several minutes. Call groups were separated from each other by an interval greater than typical intercall intervals; they invariably consisted of three distinct types of calls, which we designate as types 1, 2, and 3 (Fig. 2; Audios S1–S3). The three call types were not delivered in any specific sequential order or with regular intervals within call groups. Type 1 and type 3 calls had a pulsatile temporal structure, whereas type 2 calls were nonpulsatile (Fig. 2; Tables 1, 2). In total, 140 calls, representing 41 type 1 calls, 76 type 2 calls, and 23 type 3 calls, were recorded from three individuals (43, 50, and 47 calls/male) at the NSBG and MMNP sites and used for acoustic analyses.

Type 1. The pulsatile type 1 calls ($N = 41$) had two distinct parts: part 1 had relatively shorter and more consistent intervals between pulses, and thus a faster pulse rate; part 2 had relatively longer and less consistent intervals between pulses and thus a slower pulse rate (Fig. 2; Table 1; Audio S1). Part 1 was emitted in all measured calls, but part 2 was sometimes absent. The occasional absence of part 2 was the primary reason for the wide range of call durations (range 28.4–538.3 ms). The mean duration of the entire call was 275.8 ms, with a mean dominant frequency of 1.79 kHz (range 1.55–2.41 kHz). The maximum amplitude was reached in part 1, with a mean rise time of 7.3 ms and a very long mean fall time of 268.5 ms (Table 1).

The invariably produced part 1 was relatively short, with a mean duration of 47.0 ms (range 18.0–190.4 ms). The amplitude envelope had a mean rise time of 7.3 ms and mean fall time of 39.7 ms, with 4–12 pulses delivered at a rate of 270.8 ± 30.6 pulses/s. The mean dominant frequency measured for part 1 was 1.79 kHz (range 1.55–3.23 kHz), which was the same as that of the entire call (Table 1).



Figure 1. Calling males of *Minervarya charlesdarwinii* in the vicinity of oviposition sites. A and B, on the forest floor; C, on vegetation; D, inside a water-filled tree hole; E, inside a water-filled plastic nursery bag. Photos: S. D. Biju and G. Gokulakrishnan.

Part 2 of the call was longer in duration than part 1 but was sometimes absent ($N = 7$ of 41). Part 2 comprised 7–18 pulses delivered at a rate of 43.5 ± 7.5 pulses/s. It had a mean duration of 263.5 ms and mean rise and fall times of 27.4 ms and 236.1 ms, respectively. The mean dominant frequency of part 2 was also similar to that of the entire call at 1.86 kHz (range 1.72–2.84 kHz). Part 2 also contained the maximum amplitude pulse of the entire type 1 call, with a mean pulse period of 3.4 ms, mean pulse duration of 3.1 ms, mean pulse rise time of 1.2 ms, and a mean pulse fall time of 1.9 ms (Table 1).

Type 2. Type 2 calls ($N = 76$) comprised a single pulse having a tonal quality and lacking the distinct pulsatile temporal structure of types 1 and 3. This call type was usually produced before the type 3 calls (Fig. 2; Table 1; Audio S2). Type 2 calls had a mean duration of 78.0

ms (range 32.9–180.9 ms), mean rise time of 20.6 ms, and mean fall time of 57.4 ms. The mean dominant frequency was 2.37 kHz (range 1.46–3.10 kHz) (Table 1).

Type 3. Type 3 calls ($N = 23$) had a pulsatile temporal structure similar to type 1 calls. They had a mean duration of 644.1 ms, with a mean rise time of 59.4 ms and mean fall time of 584.8 ms. The overall mean dominant frequency was 2.29 kHz. The calls comprised 50–101 pulses delivered at a rate of 130.6 ± 16.5 pulses/s. The maximum amplitude pulse of type 3 calls had a mean pulse period of 4.3 ms, mean pulse duration of 3.9 ms, mean pulse rise time of 1.5 ms, and a mean pulse fall time of 2.4 ms (Fig. 2; Table 1; Audio S3).

Advertisement call comparison with congeners (Table 2). Of the 31 currently recognized species in the genus *Minervarya*, advertisement calls

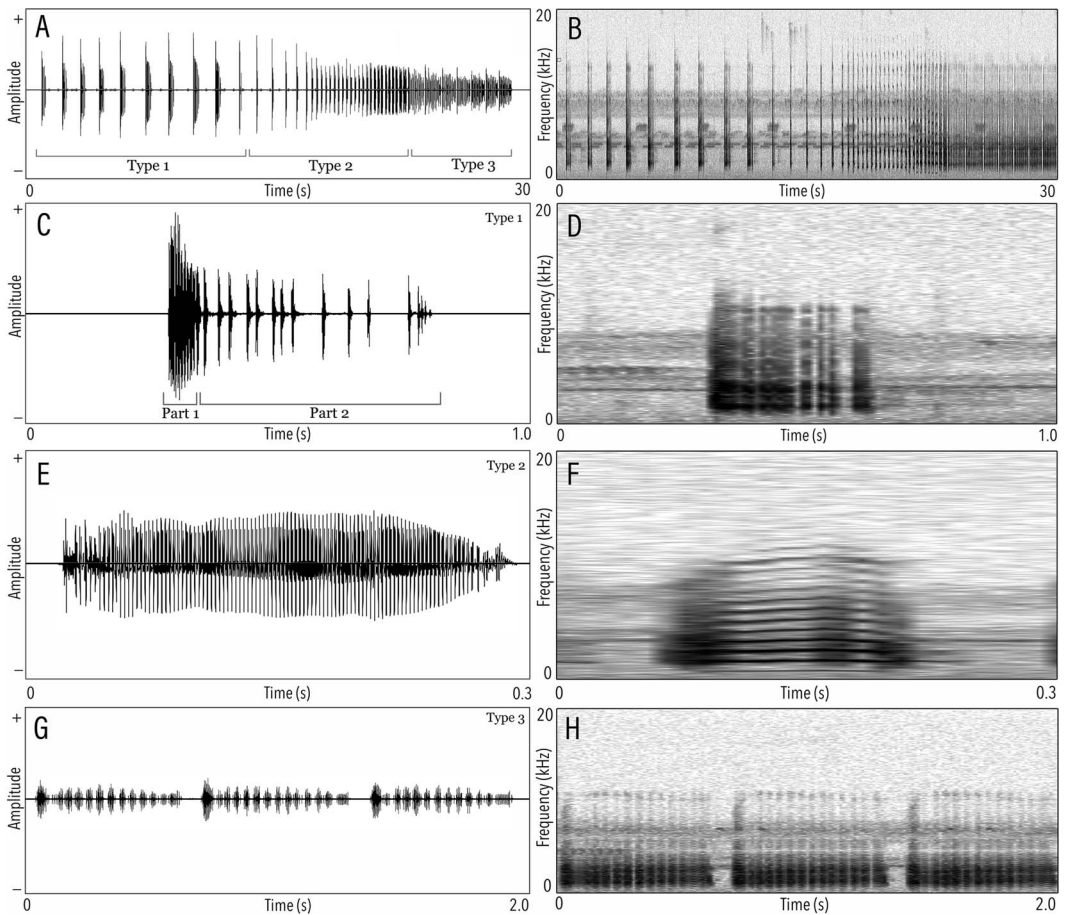


Figure 2. Male advertisement calls of *Minervarya charlesdarwini* depicted in oscillograms (left) and spectrograms (right). A and B, 30-s sections of type 1 calls, type 2 calls, and type 3 calls, delivered in groups; C and D, 1.0-s sections of type 1 calls comprised of two call parts; E and F, 0.3-s sections of type 2 calls; G and H, 2.0-s sections of type 3 calls.

are reported in the literature for 15 species. We compared these with the calls of *M. charlesdarwini*, which can be differentiated from all the others by a combination of acoustic traits that include the number of call types, call structure, and dominant frequency (Table 2). More specifically, *M. charlesdarwini* has three advertisement call types (vs. a single type in *M. agricola*, *M. chilapata*, *M. manohari*, *M. muangkanensis*, *M. mysorensis*, *M. nepalensis*, *M. nicobariensis*, *M. pentali*, *M. pierrei*, *M. rufescens*, *M. sahyadris*, *M. sahyadrensis*, and *M. teraiensis*, and two types

in *M. andamanensis* and *M. cf. keralensis*). Furthermore, type 1, type 2, and type 3 calls of *M. charlesdarwini* differ from the calls of *M. agricola*, *M. andamanensis*, *M. chilapata*, *M. manohari*, *M. mysorensis*, *M. nepalensis*, *M. nicobariensis*, *M. pentali*, *M. pierrei*, *M. rufescens*, *M. sahyadris*, *M. sahyadrensis*, and *M. teraiensis* by their lower mean dominant frequencies of 1.79 kHz for type 1, 2.37 kHz for type 2, and 2.29 kHz for type 3 calls (vs. higher mean dominant frequencies of 6, 3, 3.4, 2.6, 3.6, 2.83, 6, 3.1, 4.2, 2.9, 3.65, 3.44, and 2.45 kHz for the other species,

TABLE 1. ACOUSTIC PROPERTIES OF THREE TYPES OF MALE ADVERTISEMENT CALLS IN *MINERVARYA CHARLESDARWINI*—TYPE 1 ($N = 41$), TYPE 2 ($N = 76$), AND TYPE 3 ($N = 23$)—AND OF THE AGGRESSIVE TYPE 4 CALLS ($N = 2$).

Call Properties	Mean	SD	Minimum	Maximum
Type 1 Advertisement Call				
Entire Call				
Duration (ms)	275.8	114.6	28.4	538.3
Rise time (ms)	7.3	4.8	2.4	26.0
Fall time (ms)	268.5	113.9	23.1	531.3
Overall Dominant Frequency (kHz)	1.79	0.14	1.55	2.41
Part 1 of Call				
Duration (ms)	47.0	35.6	18.0	190.4
Rise time (ms)	7.3	4.8	2.4	26.0
Fall time (ms)	39.7	35.8	12.8	183.5
Pulses*	8	7–10	4	12
Pulse rate (pulses/s)	270.8	30.6	208.3	332.2
Overall dominant frequency (kHz)	1.79	0.14	1.55	3.23
Part 2 of Call				
Duration (ms)	263.5	60.4	150.5	367.0
Rise time (ms)	27.4	77.1	0	0.3
Fall time (ms)	236.1	81.8	3.1	367.0
Pulses*	12	10–14.3	7	18
Pulse rate (pulses/s)	43.5	7.5	30.8	60.1
Overall dominant frequency (kHz)	1.86	0.24	1.72	2.84
Maximum Amplitude Pulse of Entire Call				
Pulse period (ms)	3.4	0.4	2.6	4.2
Pulse duration (ms)	3.1	0.4	2.3	4.0
Pulse rise time (ms)	1.2	0.5	0.5	2.3
50% pulse rise time (ms)	0.5	0.3	0.1	1.3
Pulse fall time (ms)	1.9	0.6	0.7	3.0
50% pulse fall time (ms)	0.8	0.5	0.1	2.2
Type 2 Advertisement Call				
Call Properties				
Duration (ms)	78.0	33.4	32.9	180.9
Overall dominant frequency (kHz)	2.37	0.62	1.46	3.1
Pulse rise time (ms)	20.6	24.5	0.2	113.7
50% pulse rise time (ms)	3.5	3.7	0.2	11.7
Pulse fall time (ms)	57.4	33.0	20.4	160.9
50% pulse fall time (ms)	18.5	11.5	1.7	76.5
Type 3 Advertisement Call				
Call Properties				
Duration (ms)	644.1	110.8	426.1	825.8
Rise time (ms)	59.4	121.2	6.6	403.9
Fall time (ms)	584.8	163.6	235.2	807.8
Overall dominant frequency (kHz)	2.29	0.69	1.64	3.32
Pulses per call*	88.5	84–91.5	50	101
Pulse rate (pulses/s)	130.6	16.5	101.4	152.9
Properties of Maximum Amplitude Pulse				
Pulse period (ms)	4.3	1.7	3.0	9.7
Pulse duration (ms)	3.9	1.6	2.7	9.2
Pulse rise time (ms)	1.5	1.1	0.9	5.2

TABLE 1. Continued.

Call Properties	Mean	SD	Minimum	Maximum
50% pulse rise time (ms)	1.1	1.1	0.3	4.9
Pulse fall time (ms)	2.4	0.9	1.5	4.7
50% pulse fall time (ms)	1.7	0.6	0.3	2.6
Type 4 Aggressive Call				
Call Properties				
Duration (ms)	183.9	28.7	163.7	204.2
Rise time (ms)	30.7	0.8	30.1	31.3
Fall time (ms)	153.3	27.8	133.6	172.9
Overall dominant frequency (kHz)	2.78	0.91	2.71	2.84
Pulses per call*	55	48–62	48	62
Pulse rate (pulses/s)	311.4	11.0	303.6	319.2

*Because pulses are indivisible units, we report medians and interquartile ranges on the basis of individual means in place of means and standard deviations.

respectively); and differ from the calls of *M. cf. keralensis* by higher mean dominant frequencies of 1.79 kHz in type 1 and 2.37 kHz in type 2 calls (vs. 1.33 and 0.81 kHz, respectively, in *M. cf. keralensis*). The dominant frequency of type 1 calls of *M. charlesdarwini* is relatively similar to that of *M. muangkanensis* (1.79 vs. 1.82 kHz), whereas the dominant frequencies of type 2 and type 3 calls are higher than that in the single call type of *M. muangkanensis* (2.37 kHz in type 2 and 2.29 kHz in type 3 calls of *M. charlesdarwini* vs. 1.82 kHz in *M. muangkanensis*). In comparison with the dominant frequencies of type 2 and type 3 calls of *M. manohari*, those of *M. charlesdarwini* are only slightly lower, but type 2 calls of *M. charlesdarwini* can be differentiated by their non-pulsatile temporal structure (vs. pulsatile in *M. manohari*) and the slower pulse rate of type 3 calls (130.6 vs. 301.5 pulses/s in *M. manohari*). The dominant frequencies of type 2 and type 3 calls of *M. charlesdarwini* are similar to that in the single call type of *M. teraiensis* (2.37 and 2.29 kHz, respectively, vs. 2.45 kHz in *M. teraiensis*), but type 2 calls of *M. charlesdarwini* possess a non-pulsatile temporal structure (vs. pulsatile in *M. teraiensis*). Type 3 calls of *M. charlesdarwini* can also be differentiated from *M. teraiensis* calls in having more pulses per call (50–101 vs. 5–8, respectively). Finally, type 1 calls of *M. charlesdarwini* possess a unique, two-

part structure (i.e., with part 1 and part 2) that is not observed in other studied minervaryan species (Table 2).

Male vocalization when female approaches (Fig. 3; Audio S3). Upon being approached by a female, males produced only two types of calls, type 1 and type 3; they completely stopped producing type 2 calls. This suggests that type 2 calls might have a different function from type 1 and type 3 calls. We suggest three possible functions:

1. Type 2 calls could be territorial calls used to ward off other males rather than attracting females.
2. Type 2 calls could be a type of advertisement call produced to attract females but may have no role once a female approaches or selects a mate.
3. Type 2 calls could be the primary advertisement call type required to attract females, but they probably are discontinued once a female approaches or selects a mate and then are succeeded by territorial calls to ward off other intruding males and facilitate successful amplexus.

Call playback experiments may be useful in resolving the function of type 2 calls. The properties of type 1 and type 3 calls did not change in the presence of females and fell within the normal range observed in their absence (Figs. 3A, B).

Male–male acoustic interactions (Fig. 3; Audio S4). When two males interacted, the ‘resident’ (viz., most actively calling) male initially produced type 2 calls. However, between successive

TABLE 2. ACOUSTIC PROPERTIES OF MALE ADVERTISEMENT CALLS OF DIFFERENT *MINERVARYA* SPECIES (RANGE, WHEN PRESENT, IS GIVEN IN PARENTHESES).

Taxa	No. of Call Types	Call Type	Dominant Frequency (kHz)	Call Duration (ms)	No. of Pulses per Call	Pulse Rate (pulses/s)	Call Rise Time (ms)	Call Fall Time (ms)	Reference
<i>M. agricola</i>	1	type 1	6	13,000	13–16	—	—	—	Ganesh et al., 2017
<i>M. andamanensis</i>	1	type 1	3	8,000–17,000	5–8	0.66	—	—	Chandramouli et al., 2021
<i>M. charlesdarwinii</i>	3	type 1	1.79	275.8	8 & 12*	270.8 & 43.5*	7.3	268.5	Present study
		type 2	2.37	78	—	—	20.6	57.4	
		type 3	2.29	644.1	88.5	130.6	59.4	584.8	
<i>M. chilapata</i>	1	type 1	3.4	29.2–36.9	6	187.5	7.6	25.4	Gautam and Bhattarai, 2022
<i>M. cf. keralensis</i>	1	type 1	2.81–3.87	—	—	—	—	—	Ohler et al., 2009
	2	type 1	1.33 (775.20–2,239.45)	160	—	—	—	—	Hegde and Kadavevaru, 2023
		type 2	0.81 (689.06–947.46)	60	—	—	—	—	
<i>M. manohari</i>	1	type 1	2.6	260.1	76	301.5	127.2	11.7	Garg and Biju, 2017
<i>M. mutangkanensis</i>	1	type 1	1.82	330	19–25	—	—	—	Köhler et al., 2019
<i>M. mysorensis</i>	1	type 1	3.6	910	—	—	—	—	Kuramoto et al., 2007
<i>M. nepalensis</i>	1	type 1	2.83	385	56–63	—	—	—	Grosjean and Dubois, 2011
<i>M. nicobariensis</i>	2	type 1	6	150	26	—	—	—	Chandramouli and Prasad, 2020
		type 2	6	270	23	—	—	—	
<i>M. pentali</i>	1	type 1	3.1	223.1	19	83.6	35.2	22.1	Garg and Biju, 2021
<i>M. pierrei</i>	1	type 1	4.2	258	—	—	—	—	Grosjean and Dubois, 2011
<i>M. rufescens</i>	1	type 1	2.9	233.7	52	227.1	221.9	11.2	Garg and Biju, 2017
<i>M. sahyadris</i>	1	type 1	3.65	33	5–10	—	—	—	Grosjean and Dubois, 2011
<i>M. sahyadrensis</i>	1	type 1	3.44	55	9–15	—	—	—	Grosjean and Dubois, 2011
<i>M. teratensis</i>	1	type 1	2.45	85	5–8	—	—	—	Grosjean and Dubois, 2011

*Median of pulse and mean of pulse rate for part 1 and part 2 of type 1 calls, respectively.

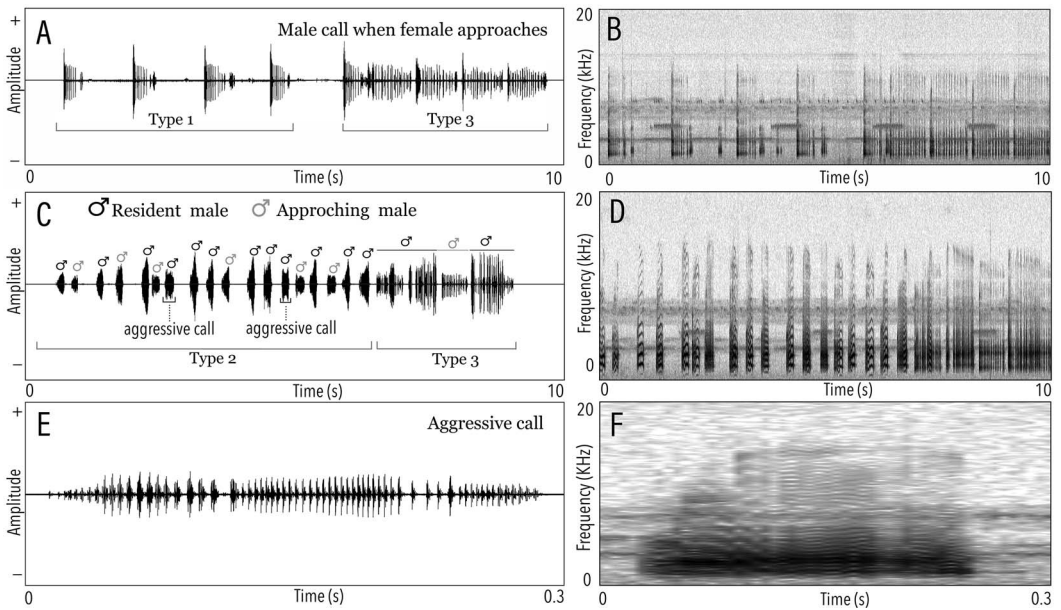


Figure 3. Calls of male *Minervarya charlesdarwini* in the presence of females and during male–male agonistic interactions, depicted in oscillograms (left) and spectrograms (right). A and B, 10-s sections of type 1 calls and type 3 calls, delivered in groups when a female approaches a calling male; C and D, 10-s sections of type 2 calls, type 3 calls, and ‘aggressive calls’ during male–male interactions; E and F, 0.3-s sections of aggressive calls.

type 2 calls a different type of call was also produced. This call cannot be assigned to any of the three types described above that are likely part of the advertisement call repertoire. We consider this additional type to be an aggressive call (Figs. 3C–F). The resident male started producing these aggressive calls an average of 4,887 ms ($N = 2$) after an invading male started producing type 2 calls nearby. The aggressive calls ($N = 2$) had a pulsatile temporal structure and a mean duration of 183.9 ms, with mean rise time of 30.7 ms and mean fall time of 153.3 ms. The calls comprised 48–62 pulses delivered at a mean rate of 311.4 ± 11.0 pulses/s. The overall dominant frequency was 2.78 kHz (range 2.71–2.84 kHz) and the spectrum had a single broad peak. As interactions between males proceeded, both males produced type 3 calls in addition to type 2 and aggressive calls. Type 1 calls were completely absent during male–male interactions. Properties of type 2 and type 3 calls did not appear to differ

substantially from the typical calls produced either to attract, or in the presence of, approaching females.

Agonistic interactions

We observed male–male agonistic behavior and attacks on amplexant pairs by unpaired males in natural populations of *M. charlesdarwini*. The following accounts are based on 27 combat sequences between males and six agonistic interactions between unpaired males and amplexant pairs at natural oviposition sites.

Male–male combat (Figs. 4A–F, M; Video S2). When a calling male detected another male approaching within close distance (< 10 cm), the static (or stationary) male emitted a short, high-amplitude vocalization with shorter intercall intervals in comparison with the advertisement calls, while simultaneously turning toward the approaching male. The aggressive

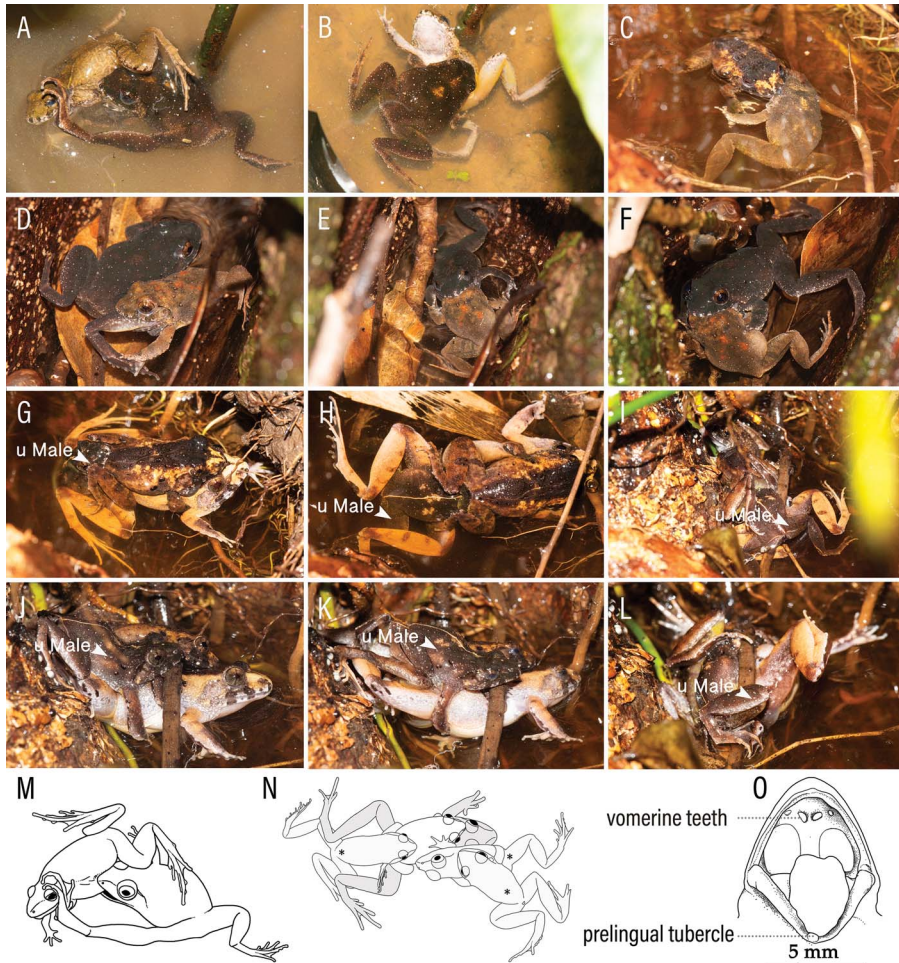


Figure 4. Physical agonistic interactions between males of *Minervarya charlesdarwinii* at the breeding sites: male-male combats (A–F) and unpaired male (“u Male”) combats with amplexant pairs (G–L). A, male kicking the head of another male with his hind limb and biting its abdomen; B, biting abdomen; C, biting head; D–F, combat sequence depicting a male first biting the hind limb (D) and then the head (E and F) of another male; G, unpaired male approaches an amplexant pair from behind (lateral view); H and I, unpaired male inserts its head between the bodies of the amplexant pair (H, dorsal view; I, posterior view); J–L, unpaired male attempts to displace the amplexant male (J, lateral view; K, dorsolateral view; L, posterior view). Arrows point to the unpaired male. M, male kicking the head of another male with his hind limb and biting its abdomen (illustration based on panel A); N, three unpaired males (*) attempting to displace an amplexant male; O, buccal cavity in a breeding male showing vomerine teeth and prelingual tubercle. Photos: G. Gokulakrishnan and S. D. Biju; drawings: S. D. Biju.

vocal interaction between the males that followed lasted up to a minute, and it ended with the static male producing a unique aggressive call (described above) that was different from all three types of advertisement calls. In all 27

observed combat sequences, the approaching male responded to the static male’s call by calling back and physical combat invariably ensued. Males exhibited three types of physical aggression:

1. Wrestling: The males clasped and pushed each other using their forelimbs and hind limbs; bouts lasted between 8 and 40 s. Wrestling was the most common form of aggression; it was seen in nearly all instances of combat and was often accompanied by the following other forms of physical aggression.
2. Kicking: Rival males kicked each other with their hind feet (4 of 27 instances, Fig. 4A).
3. Biting: Rival males grabbed a portion of the body or even the entire head of the opponent with their jaws (Figs. 4B–F, M). Biting was also frequently observed (23 of 27 instances); it usually lasted from 3 to 10 s but in three instances lasted as long as 40 s.

Males often continued to vocalize during all three types of physical aggression. These calls were audibly different from advertisement calls, but because it was not possible to distinguish between notes emitted by the two males, we did not analyze these calls.

Of the 27 male–male combat sequences, 25 were between two males; in the other two occasions, a third male was also briefly involved. After each encounter, the ‘winner’ returned to and resumed calling from almost the same site as before, whereas the ‘loser’ remained silent for up to 15 s before calling again. At least five of the 27 instances involved fights between the same two males.

Unpaired male aggression against a mated pair (Figs. 4G–L, N; Video S3). On eight occasions, we observed physical aggression by unmated males directed toward male–female amplexed pairs. In these instances, after a male achieved amplexus with a female, another partially submerged male almost immediately attacked the amplexant pair from behind and pushed his head between their bodies in an apparent effort to displace the mounted male. Of the observed events, two involved a single unpaired male, five occurred with two unpaired males, and one had three unpaired males. In none of the instances involving one or two unpaired males did they succeed in displacing the amplexant male; the mating pairs successfully achieved egg laying.

However, in the lone case that involved three unpaired males, one of them grabbed the amplexant female in her inguinal region with both forelimbs while the other two pushed their heads between the bodies of the amplexant pair (Video S3). This was the only instance in which the mated pair failed to achieve oviposition.

Upon external examination of male specimens collected both during the active breeding and nonbreeding seasons, we did not observe any bony odontoid fangs on the lower jaw (Fig. 4O). Such fangs are present in some species engaging in male–male combat (e.g., Emerson and Inger, 1992; Tsuji and Matsui, 2002). We also did not observe any external secondary sexual characters, such as prominent spines on the nuptial pads of fingers, that have been suggested to function in male–male combat (e.g., Duellman and Trueb, 1986).

Breeding sequence, amplexus and upside-down spawning

We observed differences in breeding sequence and oviposition site in *M. charlesdarwinii* between natural and unnatural microhabitats.

Breeding at natural oviposition sites (Figs. 5–8; Video S4). Natural breeding microhabitats included rain-filled tree holes or cavities in primary, disturbed, or fragmented forest areas with dominant tree vegetation. Breeding occurred predominantly in tree holes and cavities in the main trunk or branches at heights ranging from 5 cm to 1.3 m above the forest floor, in ground-level tree cavities or buttress roots 5–20 cm high, and sometimes in water collected in bamboo cuttings 5–13 cm high and in *Pandanus* leaf axils up to 1.5 m high (Fig. 5). We also observed breeding in rain-filled cavities in fallen, decomposing trees and plant parts. Although we did not document the specific species of trees used for breeding, most observations of breeding activities (both at MMNP and NSBG sites) were in native, and not cultivated, tree species. The heights of such trees ranged between 2 and 50 m and the



Figure 5. Natural oviposition microhabitats used by *Minervarya charlesdarwinii* in modified forest land at the Naya Sahar Botanical Garden site. A and B, cavities on tree buttresses; C, accumulated rainwater in the axils of *Pandanus* leaves; D, rain-filled cavity on a fallen log; E, tree hole opening 0.3 m above the forest floor. Arrows indicate standing water. Photos: S. D. Biju and G. Gokulakrishnan.

trunk diameters between 15 and 90 cm. We did not detect preference for specific tree or plant species for breeding. Water level in the cavities depended on weather conditions and the physical attributes of the cavities, such as depth, volume, and the location and orientation of the opening (Fig. 6; Table 3). The approximate size of such phytotelma openings (mostly round, oval, to elongate) ranged from 2.5 to 15 cm wide, 4.0 to 50 cm long, and 3.5 to 30 cm deep. Water depth in these reservoirs ranged from 1.3 to 15 cm. Location of the trees, either along or away from man-made trails inside forests, did not seem to affect their selection as breeding sites. However, all sites were abundantly surrounded by leaf litter on the forest

floor and by tree canopy above, suggesting that these could be important requirements for breeding and subsequent larval development. We observed four complete breeding sequences in natural sites, beginning before the initiation of amplexus through the completion of spawning, which in all instances was achieved while the pair was in an upside-down position. The following description of one sequence is based on an amplexant pair in a forest patch adjoining a managed cultivated area at the NSBG site (Table 3, event 3). A schematic representation of the breeding sequence is provided in Fig. 8, and corresponding videos are included in supplementary information.

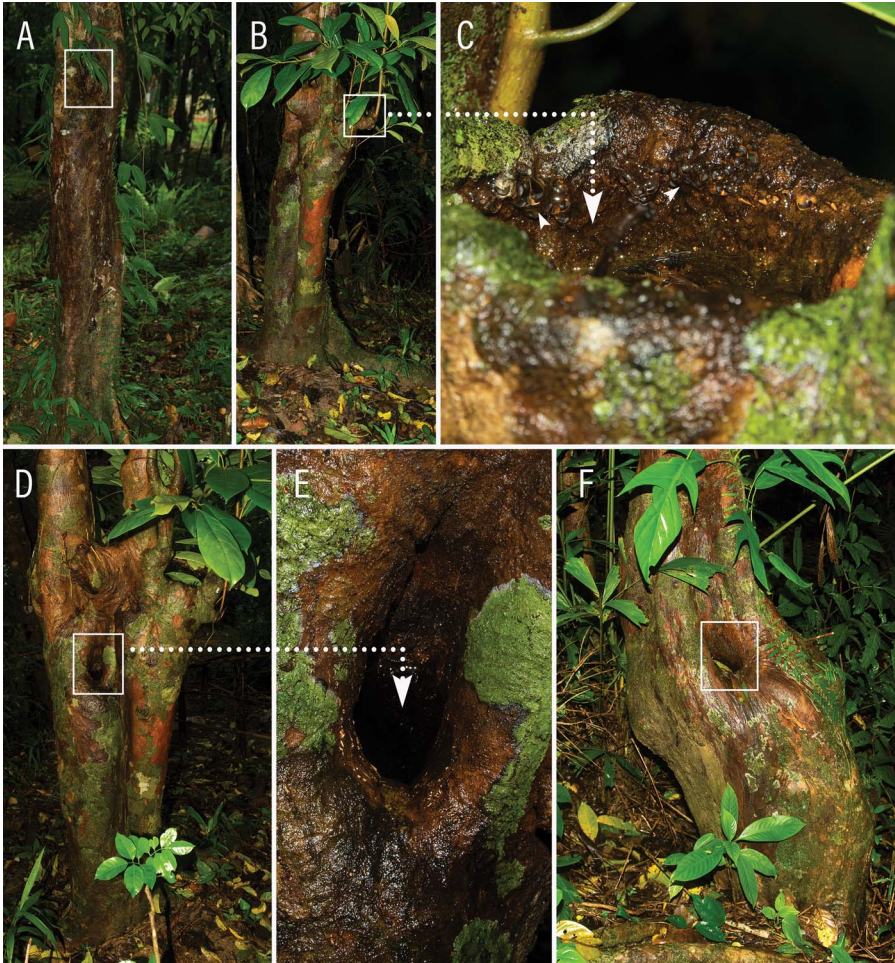


Figure 6. Natural oviposition sites in tree holes used by *Minervarya charlesdarwini* inside a primary forest. A, tree hole opening 0.6 m above ground; B, tree hole opening 0.5 m above ground; C, close-up of oviposition site in B with eggs attached to inner walls of the tree cavity above the water surface (arrowhead points to eggs); D, tree hole opening 0.4 m above ground; E, close-up of tree hole opening in D (arrowhead points to water-filled cavity); F, tree hole opening about 0.5 m above ground. Photos: S. D. Biju and Sonali Garg.

Males started calling at around 18:00 h from either the leaf litter, the close vicinity of tree cavities, or partially inside cavities (Video S5). A few males called with their bodies oriented vertically and submerged up to the forelimbs inside water-filled tree holes. After 00:30 h, two stationary females were observed near the calling males. Male vocalizations and agonistic interactions continued to 01:00 h, when a female

slowly entered the water-filled cavity and moved between two actively calling but partially submerged males. Within a few seconds, the female entered into axillary amplexus with the more actively calling male while he was still partially submerged. The amplexant pair then emerged from the water and quickly moved toward the water's edge (Figs. 7 A–C; 8 D–E). Almost immediately or even simultaneously, the other

TABLE 3. BREEDING MICROHABITATS AND BEHAVIORAL CHARACTERISTICS OF *MINERVARY CHARLES DARWINI* BASED ON EIGHT EVENTS AT NATURAL AND UNNATURAL SITES, SITE 1: FOREST PATCH AT MOUNT MANIPUR NATIONAL PARK; SITE 2: FOREST PATCH ADJACENT TO BOTANICAL GARDEN; SITE 3: BOTANICAL GARDEN NURSERY AND DISTURBED DUMP SITES.

Observation time and sites	Natural Oviposition Sites			Unnatural Oviposition Sites				
	Event 1	Event 2	Event 3	Event 4	Event 5	Event 6	Event 7	Event 8
Date/Time	18 June 2021, 2:00 h Site 1	19 June 2021, 2:30 h Site 1	12 June 2021, 1:30 h Site 2	9 June 2022, 1:50 h Site 2	11 June 2022, 2:30 h Site 3	12 June 2022, 1:20 h Site 3	28 June 2022, 1:40 h Site 3	28 July 2022, 1:50 h Site 3
Location	water-filled tree hole	water-filled tree hole	water-filled tree hole	water-filled tree buttress	water-filled plastic sap-lining bag	water-filled plastic container	water-filled automobile tire	water-filled metal container
Oviposition Site Characteristics								
Oviposition site	tree hole	tree hole	tree hole	tree buttress	plastic sap-lining bag	plastic container	automobile tire	metal container
Height of oviposition site above ground	0.3 m	0.6 m	0.4 m	0.1 m	ground	ground	ground	ground
Diameter of cavity opening	9.2 cm	11.1 cm	10.3 cm	11.4 cm	13.0 cm	8.7 cm	—	4.6 cm
Depth of cavity	5.1 cm	6.5 cm	5.7 cm	3.2 cm	5.4 cm	4.1 cm	5.3 cm	3.2 cm
Depth of water column	3.4 cm	2.3 cm	2.6 cm	2.2 cm	2.0 cm	1.2 cm	1.5 cm	1.1 cm
Breeding Behavior								
Number of individuals at the time of breeding	4 ♂, 1 ♀	5 ♂, 1 ♀	5 ♂, 2 ♀	3 ♂, 1 ♀	3 ♂, 1 ♀	2 ♂, 1 ♀	4 ♂, 1 ♀	3 ♂, 1 ♀
Male-male fight events	+	+	+	+	+	-	-	+
Unpaired male fights with amplectant pair	+	+	+	+	+	+	+	+
Oviposition and Nest Characteristics								
Number of additional upside-down turns in a single amplexus event	2	3	0	2	0	0	2	0
Total duration of amplexus	2 min 20 s	5 min 20 s	2 min 25 s	2 min 20 s	~2 min 40 s	~2 min 30 s	~2 min 50 s	~2 min 55 s
Number of egg-laying bouts	7	9	6	5	5	7	6	5
Number of eggs in individual bouts	2, 5, 6, 4, 5, 8, 4	4, 19, 5, 9, 10, 7, 6, 9, 7	4, 10, 5, 11, 6, 8	5, 9, 9, 10, 8	5, 4, 4, 7, 5	2, 4, 7, 2, 4, 4, 5	8, 7, 9, 5, 3, 6	8, 4, 5, 5, 2

TABLE 3. Continued.

	Natural Oviposition Sites				Unnatural Oviposition Sites			
	Event 1	Event 2	Event 3	Event 4	Event 5	Event 6	Event 7	Event 8
Total number of eggs laid in all bouts	34	76	44	41	25	28	38	24
Total duration of oviposition	2 min 05 s	5 min 00 s	2 min 10 s	2 min 5 s	~ 2 min	~ 2 min	~ 2 min	~ 2 min
Fertilization success rate (early embryonic development up to Gosner stage 23)	95%	96%	99%	98%	98%	100%	98%	100%

male approached the amplexant pair from behind and pushed his head between their bodies. Another calling male then joined the agonistic interaction with the amplexant pair and unpaired male. The amplexant male kicked the intruding unpaired males with his hind feet. The amplexant pair then quickly turned their bodies upside down with their heads pointing toward the water and climbed the inner walls of the tree hole in a backward movement (Figs. 7D, 8F; Video S4). The female immediately released four eggs covered with thick jelly layers in a single bout and attached them to the cavity wall, well above the water level (Fig. 7E). The intruding males continued to call and disturb the amplexant pair, but only from their front side. After about 10 s the amplexant pair shifted their position by about 20 mm from the first egg-release site. The female then deposited 10 more eggs in a second bout. In similar fashion, the pair deposited eggs in a total of six bouts (4, 10, 5, 11, 6, and 8 eggs per bout at successive intervals of about 10, 12, 18, 16, and 14 s, respectively, after the first bout), each attached separately and together forming a circular arrangement on the cylindrical cavity walls. Forty-four eggs, with gray pigment on the animal pole and an unpigmented vegetal pole, were attached to the inner wall of the tree hole 2–10 cm above the water level (Fig. 7F). Each egg measured 3.4 ± 0.3 mm in diameter including the jelly layer and 2.8 ± 0.2 mm without the layer. During egg laying in four other breeding events (of eight total), the mating pairs synchronously switched between head-up and head-down positions above and below the water surface using both forward and backward movements. The number of additional upside-down turns in a single amplexus event are provided in Table 3. On two occasions (events 1 and 4) the mating pair returned to the water after the first bout of egg laying and repeated the upside-down position after intervals of up to a minute. In general, the same tree cavities were used repeatedly as oviposition sites by multiple mating pairs at different times during the breeding



Figure 7. Breeding sequence in *Minervarya charlesdarwini*. A, male and female in axillary amplexus; B and C, amplexant pair moving forward toward the edge of a tree hole (dorsal and lateral views, respectively); D, upside-down position of the amplexant pair who are backward climbing the inner cavity walls with their heads facing down toward the water. The female deposits jelly-covered eggs in a single bout despite disturbance by a partially submerged unpaired calling male in front of the mating pair. E, close-up of the female's cloacal region at the time of egg release with the amplexing male on her back; F, freshly laid eggs attached to the inner walls of the tree cavity above the water surface. Photos: G. Gokulakrishnan and S. D. Biju.



Figure 8. Schematic sequence of breeding events and upside-down spawning in *Minervarya charlesdarwini* in and around a rain-filled tree cavity (center panel). Labels on the tree cavity locate the specific site of each event. A, male calling ~ 50 cm from the site of water collection; B, male calling from the water collection site with his entire body submerged except the head; C, female waits on a nearby substratum; D, calling male (sequence B) mounts the female in axillary amplexus; E, the amplexant pair climbs the inner walls of the water-filled tree cavity; F, the amplexant pair turns upside down with their heads facing down toward the water surface and subsequently deposits terrestrial eggs on the inner wall of the cavity in a single bout; G, developing embryos (Gosner stage 20), 50 hr postdeposition; H, young hatchlings (Gosner stage 22) inside the water-filled tree cavity. Illustrations: Natalya Zahn.

season. Apart from suggesting the limited availability of suitable oviposition sites, such a communal breeding preference may also provide protection to the terrestrial eggs from visiting predators, in contrast to unused sites, since no parental care was observed in this species.

Breeding at unnatural oviposition sites (Fig. 9). In disturbed areas adjacent to forests, where the availability of trees with water-filled tree holes and buttresses was far lower or nil, *M. charlesdarwini* bred and laid eggs in similarly small but unnatural water deposits. The small

plant nursery ($< 100 \text{ m}^2$) at NSBG was one such favored oviposition site. During the peak breeding season we observed egg clutches in up to 30 cylindrical sapling bags, each 45–130 mm in diameter. *Minervarya charlesdarwini* seems to have shifted to exploiting as breeding sites rainwater-filled or artificially watered plastic bags intended for seed germination and nurturing of young saplings in plant nurseries (Fig. 9). At both MMNP and NSBG, we also found plastic, glass, and metal containers littering the forest floor and adjacent disturbed sites that



Figure 9. Unnatural oviposition sites used by *Minervarya charlesdarwinii*. A, discarded plastic bottle; B, waste metallic automobile container with a narrow opening; C, discarded automobile tire filled with rainwater; D, glass liquor bottle; E, compressed plastic container; F, discarded metal food can; G, plastic nursery bags—inset shows three water-filled bags containing a noncalling male, a calling male, and freshly laid eggs, respectively. Arrowheads indicate the location of standing water. Photos: G. Gokulakrishnan and Sonali Garg.

were filled with water during the rainy season. These trash containers included food cans, soda and liquor bottles, automobile waste such as rubber tires and metal parts, and other kinds of empty plastic jars, bottles, or containers commonly used to hold food, detergents, chemicals, and other liquids (Figs. 9A–F). We observed calling males, developing eggs, and tadpoles inside such water-filled artificial objects, and additionally witnessed four spawning events, including in a nursery sapling bag (Fig. 9G). The breeding sequence at these unnatural sites did not vary significantly from those at natural sites, although the limited space, especially in closely packed sapling bags,

may pose atypical constraints that need further investigation. We also did not observe male–male combat in two of four mating events at unnatural sites, but unpaired males nonetheless engaged in agonistic interactions with the amplexant pairs. The number of additional upside-down turns in a single amplexus event also seemed lower at unnatural sites, although egg laying was always achieved in the head-down position. This might be due to fewer agonistic attacks on the amplexant pair by unpaired males, which in turn could be associated with the lack of sufficient space at these sites. Table 3 provides details regarding four events (5–8).

Amplexus. Amplexus in *M. charlesdarwini* is axillary and lasted from 2 min to 5 min 30 s, both at natural and unnatural breeding sites (Fig. 7A; Table 3). The preoviposition period, from the beginning of amplexus to the first bout of egg release by the female, lasted less than a minute. On the other hand, the oviposition period, which includes the entire time from the first bout of eggs to the last, including intervals for upside-down turning and repositioning while spawning, was longer, ranging from 2 to 5 min ($N = 8$). Individual bouts of egg deposition occurred in much shorter time frames of < 10 s each. Amplectant pairs separated immediately after the oviposition period; no postspawning amplexus period was observed in this species. The duration of amplexus is also shorter in *M. charlesdarwini* in comparison with other anuran species (Table 3). Despite the relatively short amplexus, we observed high fertilization rates of 95–100% (Table 3).

Tadpole necrophagy

Our study did not focus on embryonic and exotrophic development through the free-living aquatic tadpole stage in *M. charlesdarwini*, which was recently described by Gopika et al. (2023). However, and of interest, we found that tadpoles of *M. charlesdarwini* may opportunistically feed on injured or dead conspecifics, indicating the presence of tadpole necrophagy in this species (Fig. 10; Video S6).

DISCUSSION

New knowledge regarding the breeding behavior of *M. charlesdarwini* enhances our understanding of how this species interacts with its environments and which habitats are essential for its survival. The unique and specialized traits that we report here also yield insights into the behavioral diversity in the genus, as well as the evolution of reproductive modes in anurans generally. Finally, knowledge



Figure 10. Necrophagy by tadpoles of *Minervarya charlesdarwini*. A and B, Live tadpoles (~ Gosner stage 32) opportunistically feeding on a dead conspecific metamorph. Photos: G. Gokulakrishnan.

of *M. charlesdarwini*'s breeding requirements will serve as important considerations for conservation assessment and population monitoring of this threatened species, which is classified as Vulnerable B1ab(iii) in the most recent IUCN Red List assessment (IUCN, 2023).

The following combination of traits makes reproduction in *M. charlesdarwini* unique:

1. Males produce at least three different types of complex mating calls, as well as an aggressive call.
2. Unpaired males engage in agonistic interactions with each other and with amplectant pairs.
3. Mate selection, amplexus, and oviposition occur within water-filled cavities.
4. Mating pairs display multifaceted positions, ranging from an initial head-up orientation when their bodies are submerged to a unique upside-down position above the water surface at the time of egg laying.
5. Oviposition is quick and achieved in the upside-down position, with the heads of the amplectant pair pointing down toward the water.
6. Large eggs individually enclosed by a jelly layer are released from the female's cloaca and attached directly to the inner walls of water-filled tree cavities or artificial substratum, above the water surface.

7. Egg laying is achieved in multiple bouts of < 20 eggs each, and multiple egg masses are separately attached in a circular formation on the cylindrical inner walls of the cavities.

In addition, two other confirmed observations add to the uniqueness of this species (Gopika et al., 2023):

1. Developing embryos remain adhered to the walls of the cavities until Gosner stage 23, when they hatch and naturally drop into the water-filled cavity below.
2. Further development is completed as free-swimming exotrophic larvae inside the water-filled cavities.

The reproductive behavior of *M. charlesdarwini* is unique within the family Dicroglossidae, and perhaps among anuran amphibians, primarily owing to two traits:

1. Breeding and oviposition inside water-filled tree cavities and phytotelmata, with terrestrial eggs being deposited above the water surface.
2. Vertically upside-down egg-laying posture of the mating pair, whose bodies are completely out of the water at the time of egg laying.

Reproductive modes in the family Dicroglossidae are diverse, especially in terms of oviposition site preference and development. Most dicroglossid genera have aquatic oviposition in permanent or temporary open bodies of water, such as shallow stagnant pools, ponds and ditches (lentic), or the edges of flowing streams (lotic). These include several in the subfamily Dicroglossinae: *Chrysopaa* (e.g., Khan and Ahmed, 1987; Hofmann et al., 2023); *Euphlyctis* (e.g., Mohanty and Dutta, 1977; Tabassum et al., 2011; Chowdhury et al., 2021); *Fejervarya* (e.g., Stuart et al., 2006; Vitt and Caldwell, 2013; Lin et al., 2020); *Hoplobatrachus* (e.g., Tabassum et al., 2011; Channing et al., 2012; Lin et al., 2020); *Minervarya* (e.g., Dubois et al., 2001; Kuramoto et al., 2007; Grosjean and Dubois, 2011; Garg and Biju, 2017, 2021; Gopika et al., 2023; Hegde

and Kadadevaru, 2023), except *M. charlesdarwini* (Das, 1998; Gopika et al., 2023); *Sphaerotherca* (e.g., Mohanty et al., 1979; Sajjan et al., 2017); *Nanorana* (e.g., Lu et al., 2016; Wang et al., 2017; Shrestha and Gurung, 2019; Batool et al., 2023); *Quasipaa* (e.g., Liu and Hu, 1975; Karsen et al., 1998; Ngo et al., 2013; Lin et al., 2020); and presumably *Ombrana* (e.g., Shrestha and Gurung, 2019; Wangyal et al., 2021). Both aquatic and terrestrial oviposition have been observed in at least two genera, *Limnonectes* and *Occidozyga* (see examples below), belonging to two different currently recognized subfamilies Dicroglossinae and Occidozyginae, respectively. The reproductive mode in *Nannophrys* differs from other members of the family in having both oviposition and tadpole development in terrestrial to semiterrestrial environments. Eggs are laid in wet rock crevices along flowing streams or roadcuts, attached in one or two layers to the bottom surface of the crevice containing a thin film of water, and subsequently hatch into semiterrestrial tadpoles (Wickramasinghe et al., 2004). Species of the genus *Ingerana* (subfamily Occidozyginae) are likely stream breeders—amplectant pairs have been observed at the edges of streams—but no eggs or larvae have been documented (Ming, 2005; Sailo et al., 2009), leaving the oviposition mode of this group largely unknown.

The diversity of reproductive modes among dicroglossids to date is highest in the genus *Limnonectes*, including *L. larvaepartus*, which is unique in having internal fertilization, intraoviductal larval maturation, and the birth of free-swimming tadpoles (Iskandar and Tjan, 1996; Iskandar et al., 2014). Among other congeners, many prefer terrestrial oviposition sites and some lay eggs in aquatic environments, both lectic and lotic; after hatching, however, tadpoles complete development and metamorphosis in aquatic habitats (e.g., Tsuji and Lue, 1998; Brown and Iskandar, 2000; Tsuji, 2004; Das et al., 2007; Inger and Stuart, 2010; Rowley and Altig, 2012; Lin et al., 2020; Frederick et al., 2023). Moreover, members of this

genus display a variety of oviposition sites. These include shallow depressions in stream beds in *L. leporinus* and *L. malesianus* (Emerson, 1992; Emerson and Inger, 1992; Inger and Stuebing, 2005), *L. poilani* (Orlov, 1997), and *L. pseudodoriae* (Yodthong et al., 2021); under leaf litter on the forest floor in *L. limborgi* (Rowley and Altig, 2012), *L. hascheanus* (Inger and Stuart, 2010; Rowley and Altig, 2012; Frederick et al., 2023), and *L. arathooni* (Brown and Iskandar, 2000; Frederick et al., 2023); moist rock platforms above shallow pools in *L. woodworthi* (Binaday, 2018); and rock surfaces or overhanging vegetation (such as leaves, roots, and wood vines) along stream banks in *L. modestus* (Lubis et al., 2008), *L. leytenis* (Maglangit et al., 2020), *L. cf. visayanus* (Decena et al., 2020), and *L. phyllofolia* (Frederick et al., 2023). Some species, such as *L. blythii*, occasionally choose a variety of substrates for oviposition primarily in aquatic environments, such as in shallow areas of slow-flowing or almost stagnant streams, as well as sand, gravel stream beds, and dead organic matter, leaves, and roots from overhanging vegetation floating in streams (e.g., Dring, 1979; Emerson and Inger, 1992; Sridhar and Bickford, 2015). In *L. kuhlii*, eggs are scattered just above the surface of streams or ditch beds and become attached to submerged leaf litter (Tsuji and Lue, 1998). Finally, terrestrial oviposition under leaf litter and transportation of tadpoles by male parents to nearby water bodies, which may include cavities in fallen trees, are reported in *L. finchi* and *L. palavanensis* (Inger, 1985; Inger et al., 1986; Inger and Voris, 1988; Goyes Vallejos et al., 2018), and tadpole transportation has also been observed in *L. parvus* (Lama and Senarillos, 2023).

Members of the genus *Occidozyga* are known to breed and lay eggs in a variety of aquatic sites. These sites include, for example, flooded depressions in the forest floor and shallow silty ground puddles along forest trails, both often filled with some leaf litter, for *O. baluensis* (Haas et al., 2014); isolated pools of water along riparian zones for *O. laevis* (Decena et al., 2020); and

presumably around shallow creeks for “stream forms” such as *O. diminutiva* (Chan et al., 2020). Terrestrial oviposition is known for *O. sumatрана*, where females deposit eggs outside water on the ceiling of muddy ground hollows along small channels and streams surrounded by rice fields (Eto and Matsui, 2012). In another recently described species, *O. shiwandashanensis*, eggs were reportedly found attached to plant roots close to but not in water near muddy areas, presumably along streams, restricted to forested areas (Chen et al., 2022).

To the best of our knowledge, no dicroglossid species other than *M. charlesdarwinii*, as reported here, naturally breeds as well as deposits terrestrial eggs inside water-filled tree cavities or phytotelmata, where further development of embryos and tadpoles takes place. Furthermore, *M. charlesdarwinii* spawns in an upside-down position that is rarely seen in terrestrially breeding anurans and unique to this species in many ways. This behavior somewhat resembles the spawning position of a few aquatic-breeding frogs, such as the African pipid *Hymenochirus boettgeri*, in which oviposition is accomplished in an “upside-down” position at the water surface (Rabb and Rabb, 1963a). In *Phrynobatrachus latifrons*, another African species, the submerged amplexant pair orients upside down while spawning and the female deposits a single layer of eggs spread over the water surface (Rödel, 2000; AmphibiaWeb, 2024). In aquatic-breeding *Occidozyga sumatрана*, eggs are deposited on the ceiling of muddy hollows above the water at stream edges while the female inclines her head down and elevates her pelvic region; the amplexant pair then stands on fully stretched hind limbs (Eto and Matsui, 2012). In another aquatic-breeding Indian frog *Nyctibatrachus kumbura*, the female alone performs a similar kind of “headstand” while laying eggs above flowing streams once the amplexant male has dismounted (Gururaja et al., 2014). In another dicroglossid, *Limnonectes kuhlii* from Indonesia, amplexant pairs assume a “handstand-like spawning posture” while remaining in shallow,

gently flowing water, and then lay eggs on the water surface that get scattered or attached to submerged material (Tsuji and Lue, 1998). Finally, *Minervarya andamanensis*, the sister species of *M. charlesdarwinii*, breeds in open water bodies, and at the time of egg laying, the female lowers her head into the water while raising her cloaca toward the water surface; the male simultaneously arches his body to push the eggs close to his cloaca, causing them to be fertilized before they are scattered over the water surface (Gopika et al., 2023). However, most instances of downward movement or partial submergence of the head appear to function only to raise the cloaca so that eggs are deposited at the water surface. Hence, they exemplify a more generalized spawning style seen in many aquatic-breeding species, including microhylids (Garg et al., 2018), and may not represent a truly upside-down spawning posture in terrestrial microhabitats. In some phytotelm-breeding hylids, the amplexant pairs assume an upside-down posture at the time of oviposition while still being submerged in water. For example, in certain members of the *Scinax perpusilla* (= *Oloolygon perpusillus*) species group, such as *S. litoreus*, *S. perpusillus*, and *S. v-signatus*, amplexant pairs assume an upside-down position and lay eggs on the water surface or at the edge of the pool of water in bromeliads (Alves-Silva and da Silva, 2009), whereas in *Anotheca spinosus* (= *Tripriion spinosus*), eggs are laid just above the water surface and the pair remains partially submerged with only their cloacae above it (Jungfer, 1996). In *Minervarya charlesdarwinii*, the amplexant pair together assumes a completely vertical, head-down position with their bodies well above the water surface and then lays terrestrial eggs, a behavior that differs in many respects from instances of head-down spawning seen in other frogs. We suggest that the upside-down spawning behavior of *M. charlesdarwinii* may be a means of preventing aggressive unpaired males from displacing the amplexant pair from behind and disrupting egg laying. We observed less aggression

from unpaired males once the amplexant pair had turned and assumed a head-down position, which may constitute a defensive posture or even a form of threat. Future detailed studies are needed to understand the pattern of evolution of such behaviors, including whether they represent convergence or the retention of a primitive trait during the evolution of a novel terrestrial specialization.

In addition to oviposition and embryonic and larval development, *M. charlesdarwinii* also utilizes arboreal, water-filled tree cavities for vocalizing, mate selection, and amplexus. Both sympatric congeners, *M. andamanensis* and *M. agricola*, breed in temporary water bodies; females lay a single layer of eggs that forms a thin film on the surface of small natural or artificial ponds and flooded agricultural fields. In contrast, all breeding sites of *M. charlesdarwinii* that we observed were located inside forests with large trees or in disturbed sites contiguous to forests. Thus, *M. andamanensis* and *M. agricola* are sympatric with *M. charlesdarwinii*, but not syntopic. We hypothesize that the specialized behavior of using tree cavities for breeding and oviposition in *M. charlesdarwinii* is an adaptation that facilitates microhabitat partitioning among these co-occurring species. Another anuran species in the Andaman Islands, the bufonid *Blythophryne beryet*, breeds in phytotelmata, possibly because of the lack of small water bodies, especially in forested regions (Chandramouli et al., 2016). *Blythophryne beryet* occurs in sympatry with another bufonid, *Duttaphrynus* sp., but there are no data regarding possible niche partitioning by the two species. Overall, these findings highlight the diversity of reproductive behaviors (e.g., Das, 1998; Chandramouli et al., 2016; Biju et al., 2020; Gopika et al. 2023) even among the small number of anuran amphibians found on the Andaman Archipelago.

Studies on many tropical, subtropical, as well as temperate anurans report multiple mating and oviposition events by individual female frogs during a single breeding season (Duellman and Trueb, 1986; Wells, 2007). Examples include *Bombina*

variegata (Bombinatoridae—Smith, 1969; Barandun et al., 1997); *Colostethus inguinalis* and *Oophaga pumilio* (Dendrobatidae—Wells, 1980; Pröhl and Hödl, 1999); *Eleutherodactylus coqui* (Eleutherodactylidae—Townsend and Stewart, 1994); *Boana rosenbergi*, *Dryophytes chryso-scelis*, *D. cinereus*, *D. gratosus*, *Pseudacris regilla* (Hylidae—Kluge, 1981; Perrill and Daniel, 1983; Ritke et al., 1990); *Hyperolius marmoratus* (Hyperoliidae—Telford and Dyson, 1988); *Aquarana clamitans* (Ranidae—Wells, 1976); and *Polypedates leucomystax* (Rhacophoridae—Sheridan, 2009). Among dicroglossids, female *Limnectes kuhlii* lay multiple egg clutches in a single season and may mate with a different male each time (Tsuji and Lue, 2000); female *L. palavanensis* and possibly other female congeners may also lay multiple egg clutches in a single season (Inger et al., 1986; Inger and Voris, 1988; Tsuji and Lue, 2000). Finally, there are reasons to believe that female *Minervarya charlesdarwini* also may breed multiple times in a single season. First, other minervaryan species in the region and of similar snout–vent length deposit many more eggs—up to 150—in a single mating, which may reduce their likelihood of multiple breeding events in the same season (unpublished data). Second, one female *M. charlesdarwini* that we captured immediately after oviposition was found to have retained nearly 30 mature ova, which suggests that not all the eggs produced in a given breeding season are released in a single mating event.

The duration of amplexus ranges widely among anuran amphibians, from 5 min in *Nyctibatrachus humayuni* (Williaert et al., 2016) to more than a month in *Atelopus varius* (Crump, 1988). Amplexus in *Minervarya charlesdarwini* is relatively short lived, which may help to minimize agonistic encounters from unpaired males and quickly achieve egg laying and fertilization. The same hypothesis has been proposed to explain short amplexus durations in *Nidirana adenopleura* (as *Rana adenopleura*) in Taiwan (Chuang et al., 2013). In *Minervarya andamanensis*, the sister

species of *M. charlesdarwini*, amplexus can last up to 2–3 hr and aggressive agonistic encounters are not known to occur to such extents; the latter is also the case in other congeners. Furthermore, postspawning amplexus, which may enhance the likelihood of fertilization, is seen in some species that lay eggs in multiple bouts (Tsuji and Lue, 1998; Wells, 2007). However, although we observed female *M. charlesdarwini* to lay eggs in multiple bouts, each amplexant pair separated immediately after oviposition and none had gotten back together while we observed them continuously for up to 30 min. Our preliminary data suggest fertilization success rates > 95% despite the absence of postspawning amplexus between a given mated pair (Table 3).

Among anuran amphibians, agonistic interactions occur in a myriad of forms and intensities, either among males (Wells, 1977; Arak 1983; Heying, 2001; Caldwell et al., 2010; Costa et al., 2020) or when unpaired males attack mated pairs and attempt to displace males already in amplexus (Davies and Halliday, 1978; Tsuji and Lue, 1998; Lu et al., 2009; Chuang et al., 2013). Aggressive male–male encounters are common, especially in explosively breeding species. These encounters largely include aggressive vocal interactions or physical combat. Physical combat usually begins when vocalizations fail to ward off intruders (Martins et al., 1998; Wells, 2007). Aggressive behaviors such as wrestling (Wells, 2007; Caldwell et al., 2010), biting (Rabb and Rabb, 1963b; Brattstrom and Yarnell, 1968; Weygoldt, 1981; Giddings, 1984; Townsend et al., 1984; Katsikaros and Shine, 1997), or kicking (Telford, 1985) can last from a few seconds to several minutes. Territoriality and male agonistic behavior in anurans are mainly associated with competition for reproductive resources (Wells, 1977). In our study, the extremely intense agonistic interactions among males of *M. charlesdarwini* and between unpaired males and mated pairs appear to be related to the higher density

of males compared with females, as well as the fact that the same calling sites are used for courtship, amplexus, and oviposition. In some instances of male–male combat, however, it was difficult to differentiate resident from intruding males on the basis of direct observation, as is otherwise possible in prolonged breeders that may have more stable territories or calling sites. In *M. charlesdarwinii*, all the males at an oviposition site seemed to reside in close vicinity during the breeding season (including daytime). Thus, additional studies are needed to confirm territoriality in this species. Wells (2007) reported physical combat in at least 14 anuran families (which corresponds to 29 families in the current, revised taxonomy; Frost, 2024), including a cursory report of male–male fights in a species of *Minervarya* from Peninsular India (Kanamadi et al., 1995). The agonistic behaviors we describe in *M. charlesdarwinii* indicate that male–male combat may be more widespread in the genus and deserving of future investigation.

This study presents the first report and description of male calls in *M. charlesdarwinii*. The species' vocal repertoire consists of three types of potential advertisement calls and an aggressive call, each with distinct acoustic properties that vary on the basis of social context. Although this call pattern does not resemble any documented calls in other minervarian species, complex vocal repertoires are found across a taxonomically broad range of anurans (Schwartz, 1987; Narins et al., 2000; Christensen-Dalsgaard et al., 2002; Feng et al., 2002; Larson, 2004; Chuang et al., 2016) and the use of different call types commonly varies with social context (reviewed by Gerhardt and Huber, 2002; Toledo et al., 2015). We hypothesize that types 1, 2, and 3 calls represent the advertisement call repertoire in *M. charlesdarwinii* and that these three call types may have different communicative functions, different intended receivers, or both, as demonstrated in playback experiments conducted in other species (e.g., Narins and Capranica, 1978).

Testing this and related hypotheses about call functions in *M. charlesdarwinii* will require additional observations and playback experiments. Future investigations of the vocal repertoires in other species of *Minervarya* would also provide insights into the evolution of acoustic communication in the genus.

At least five species of phytotelm-breeding frogs with free-swimming (exotrophic) tadpoles are known from India: *Bufoides kempii* (Naveen et al., 2023), *Blythophryne beryet* (Chandramouli et al., 2016), *Uperodon anamalaiensis* (Inger et al., 1984; Garg et al., 2018), *U. montanus* (Krishna et al., 2004; Krishna and Bosch, 2007; Garg et al., 2018), and *Minervarya charlesdarwinii* (Das, 1998; Gopika et al., 2023; present study). Whereas dedicated natural history studies will likely reveal more surprises from this understudied region, it is also important to understand the drivers of this reproductive mode. Phytotelm breeding is a specialized form of terrestrial egg laying, which itself is an extraordinary derived reproductive trait in anuran amphibians despite having evolved multiple times (Gomez-Mestre et al., 2012). Even though deposition of terrestrial eggs may have evolved as an adaptation for protection against aquatic predators, a clutch with a reduced number of eggs that need certain climatic conditions to survive poses additional challenges. Over and above such trade-offs, *M. charlesdarwinii* also shows a combination of complex behaviors, including intense competition among breeding males for females as well as limited availability of favorable oviposition sites. At the same time, communal breeding within the same tree cavities could be advantageous in protecting eggs from predators, especially since parental care is not known in this species. We also observed tadpole necrophagy, suggesting that food sources may be limited. However, we did not find any evidence of oophagy or cannibalism, which are frequently observed in species that breed in ephemeral water bodies with limited external food sources, such as tree holes (Lannoo et al.,

1987; Crump, 1992; Jungfer, 1996; Caldwell and Araújo, 1998; Babbitt and Meshaka, 2000; Biju et al., 2016).

Minervarya charlesdarwini appears to obligately breed in rain-filled, naturally occurring phytotelmata. Protection of these specialized and vulnerable microhabitats will be important to maintain adequate availability of natural breeding sites and likely the long-term survival of the species. The most common natural breeding sites we observed were water-filled cavities in the main trunk or buttress roots of large native trees of the Andamans. Hence, measures to ensure sufficient suitable habitats for viable populations of *M. charlesdarwini* would likely benefit from efforts to conserve native tree species. An indirect inference could be derived from our choice of study sites, which had the largest numbers of breeding individuals observed during preliminary surveys. Two of these sites are part of existing in situ and ex situ conservation efforts. The MMNP is a national park protected under the Government of India's Wildlife Protection Act 1972. On the other hand, NSBG is an experimental garden-cum-arboretum managed by the Botanical Survey of India, which offers in situ protection to native trees while also serving as an ex situ site for cultivation of rare and threatened plant species. The higher numbers of breeding individuals at these sites likely indicate the positive impacts of forest conservation on the breeding population of *M. charlesdarwini*. Indeed, these protected sites may be serving as refuges for the remaining naturally breeding populations of Charles Darwin's frog. Future research can focus on gathering the quantitative data needed to support this hypothesis. Additional studies are needed to quantitatively test this hypothesis and to evaluate possible differences in breeding success and population viability between natural and unnatural breeding sites.

Utilization of unnatural sites for breeding may be one way this species is adapting to potential changes driven by reduced availability of tree holes, the forests themselves, and associated climatic factors, while also signaling that

things are not right in its natural breeding environments. Even though altered behaviors could be helping this species to survive, they may not ensure that the populations of Charles Darwin's frog can thrive in the long term with increasing human dominance and rapidly changing landscapes, which are making these islands less remote than they once were.

Our findings point to the relevance of reproductive biology to anuran conservation. Both the availability and the quality of natural breeding habitats affect the population size of *M. charlesdarwini*; considering them can help predict potential threats associated with land-use changes and enhance assessments of the species' conservation status. The first Global Amphibian Assessment categorized *M. charlesdarwini* as Critically Endangered (GAA1—IUCN, 2004), primarily because of the small number of threatened populations known at that time. Several additional populations throughout the Andaman Islands have been documented in subsequent years (e.g., Chandramouli, 2017; Garg et al., 2022), and the corresponding increase in the extent of occurrence of *M. charlesdarwini* has led to the recent downlisting of its conservation status to Vulnerable B1ab(iii) (GAA2—IUCN, 2023). However, new detailed knowledge of the species' breeding requirements suggests the need for additional conservation assessment on the basis of the availability of natural breeding sites and the size of breeding populations at all documented localities. At the same time, protection of conspecific populations that lie outside protected areas should be prioritized in conservation planning for this species.

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SUPPLEMENTAL MATERIAL

The below list of supplemental material referenced in this paper is available online at <https://doi.org/10.3099/0006-9698-577.1.1>.

Audio S1. Type 1 male call of *Minervarya charlesdarwini* (0.3 sec segment)

Audio S2. Type 2 male call of *Minervarya charlesdarwini* (0.3 sec segment)

Audio S3. Type 3 male call of *Minervarya charlesdarwini* (0.7 sec segment)

Audio S4. Male call of *Minervarya charlesdarwini* in the presence of an approaching female (10 sec segment)

Audio S5. Male-male acoustic interaction in *Minervarya charlesdarwini* (10 sec segment)

Audio S6. Aggressive call of *Minervarya charlesdarwini* (0.3 sec segment)

Video S1. Male *Minervarya charlesdarwini* producing advertisement calls

Video S2. Male-male combats in *Minervarya charlesdarwini*

Video S3. Unpaired male aggression against an amplexant pair in *Minervarya charlesdarwini*

Video S4. Upside-down spawning in *Minervarya charlesdarwini*

Video S5. Oviposition site in *Minervarya charlesdarwini*

Video S6. Tadpole necrophagy in *Minervarya charlesdarwini*

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