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Not enough time: short-term female presence after oviposition does not improve egg survival in the emerald glass frog

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In species exhibiting egg attendance, parents remain with their eggs, protecting them against harsh abiotic conditions, such as dehydration or drowning, and biotic conditions, such as predation, parasitism and diseases. This form of postoviposition parental care is widely observed in animals, including amphibians. Long-term egg attendance (spanning several days) is common among glass frogs, and removal experiments have demonstrated the critical role of the parent, whether male or female, in increasing egg survival. However, in a few glass frog species, females stay close to their eggs for less than 3 h after oviposition. Previous studies have found that maternal presence reduces dehydration and predation despite the short duration of this attendance behaviour. In the emerald glass frog, *Espadarana prosoblepon*, females remain close to their eggs for less than 1.5 h after oviposition. Given that the embryonic development period in this species spans an average of 25 days, our main question was whether remaining with the clutch for only 0.15% of this time is sufficient to increase egg survival. To address this question, we conducted a female removal experiment in semicaptivity and found no evidence that female presence improved egg hydration after oviposition. In a maternal commitment behavioural assay, most females promptly abandoned their clutches when subjected to a gentle disturbance and did not return to them. Lastly, a female removal experiment under field conditions revealed that clutches with and without mothers experienced similar levels of mortality, primarily caused by invertebrate predators. Through a series of experimental assays, we demonstrate that the short-term female presence in *E. prosoblepon* did not increase egg survival. Therefore, we argue that the postoviposition behaviour observed in this species cannot be considered parental care behaviour. Our findings challenge the assumption that the proximity of parents and their eggs is an unequivocal indicator of parental care.

In many animal species, females, males, or both engage in parental care, and the evolution of such behaviour has been the focus of intense theoretical and empirical investigations in behavioural ecology (Royle et al., 2012). Theoretical models suggest that different factors may influence the evolution of parental care, including sex ratio, adult mortality, paternity certainty, environmental variability and reproductive value of offspring (see Figure 2.2 in Klug et al., 2012). However, all models concur that parental care is advantageous only when the benefits to the offspring outweigh the costs incurred by the parents. Considering the great diversity of parental behaviours in nature, the benefits and costs associated with different forms of parental care are expected to exhibit substantial variation across taxa.

Among ectotherms, egg attendance is perhaps the most prevalent form of postoviposition parental care (Smiseth et al., 2012). From oviposition and during embryonic development, parents remain with their eggs, providing protection against harsh abiotic conditions, such as dehydration or drowning, and biotic conditions, such as predation, parasitism and fungal infection (reviewed in Alonso-Alvarez & Velando, 2012). Furthermore, empirical evidence from diverse taxa strongly supports the notion that egg attendance increases egg survival. In fact, field studies across several taxa have demonstrated increased egg mortality when parents are experimentally removed from their clutches (e.g. arthropods: Santos et al., 2017; fish: Goldberg et al., 2020; frogs: Machado &

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Macedo-Rego, 2023). Collectively, these studies highlight the crucial role of parental presence in benefiting the offspring.

Various biotic and abiotic conditions may influence the costs paid by the caregivers, leading to adjustments in the amount of care provided to the eggs. For instance, parents may need to leave their eggs temporarily unattended to find food (e.g. Rangeley & Godin, 1992; Requena et al., 2012; Wickramasinghe et al., 2004) or to find refuge when predation risk is high, thus reducing the time they spend with their eggs (e.g. Chuang et al., 2017; Gravolin et al., 2021). Likewise, fluctuations in abiotic conditions, such as temperature and humidity, can influence the ability of parents to remain with their eggs. For example, when protecting the offspring comes at the expense of individual water loss, parents may temporarily abandon their eggs to avoid dehydration (e.g. Chelini & Machado, 2012; Consolmagno et al., 2016). Therefore, the decision-making process for parents involves navigating the balance between self-preservation and ensuring offspring protection.

In anurans, egg attendance is widespread and has independently evolved multiple times (Furness & Capellini, 2019). During egg attendance, parents remain with or near their eggs for extended periods, sometimes until hatching commences (e.g. Puerto Rican cave-dwelling frog, *Eleutherodactylus cooki*: Burrowes, 2000; green-striped glass frog, *Hyalinobatrachium talamancae*: Chaves-Acuña et al., 2020; smooth guardian frog, *Limnonectes palavanensis*: Goyes Vallejos et al., 2018; Fig. 1a). By attending the eggs, parents provide protection against abiotic and biotic factors, mainly protection against dehydration and predation (Machado & Macedo-Rego, 2023). Although the costs of egg attendance are not so intensively studied in anurans, the body condition of the parents may decrease during the caring period, either due to increased energy expenditure while tending to the eggs or decreased foraging frequency (Machado & Macedo-Rego, 2023). Moreover, because amphibians have permeable skin, remaining with their eggs for long periods may expose the parents to water loss when the clutch is laid in places exposed to sunlight (e.g. Consolmagno et al., 2016).

As a strategy to minimize the costs of parental care, parents in some frog species may temporarily or permanently abandon their

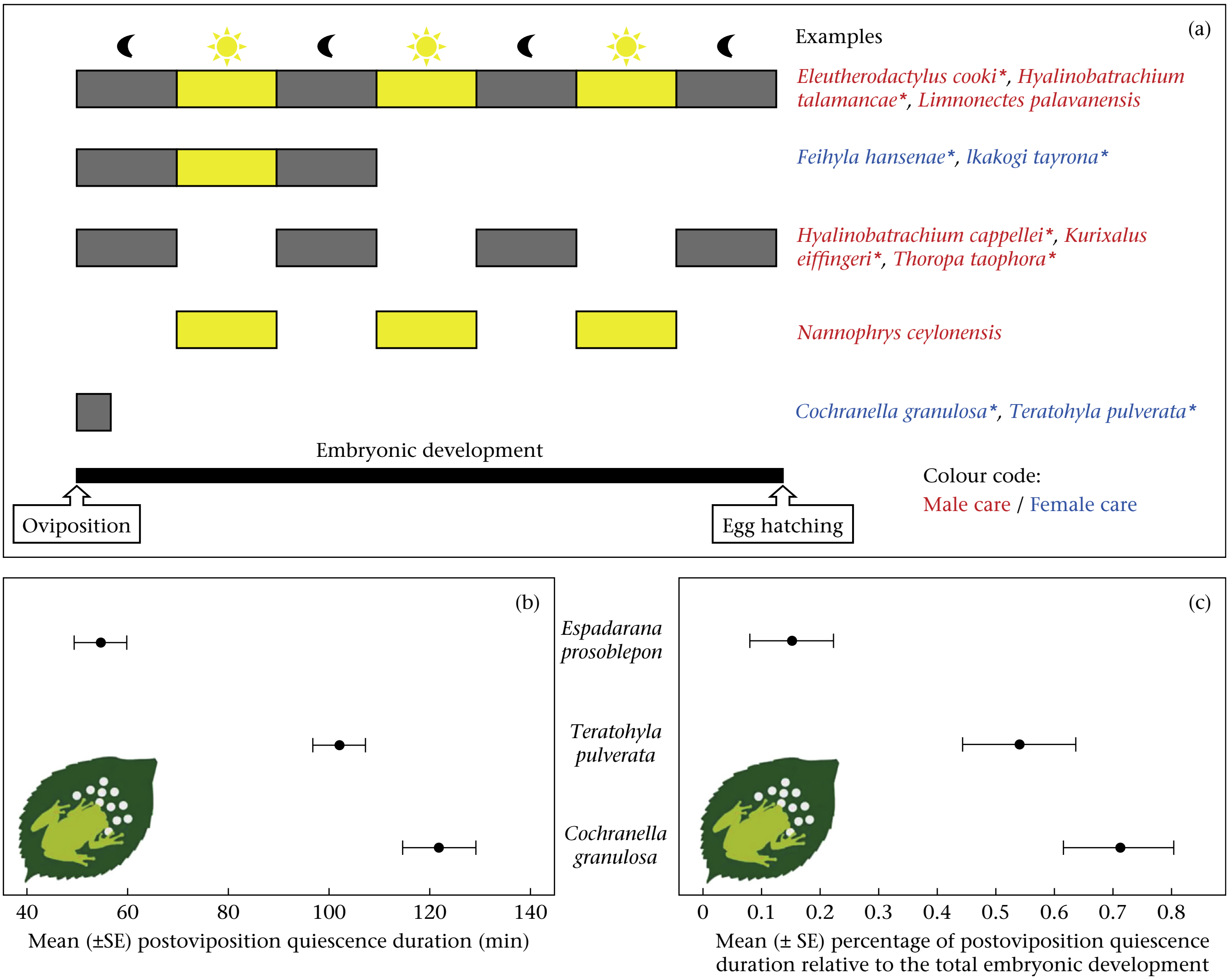

Figure 1. (a) Schematic representation of the time of permanence of the parents (males or females) close to their eggs during the period of embryonic development (i.e. number of days from oviposition until hatching) in some frog species with parental care. Certain species care for the eggs most of the time, while others care for the eggs only during the day (indicated by the sun and yellow rectangles) or during the night (indicated by the moon and grey rectangles). There are also species that care for the eggs only for a few days after oviposition and then abandon the clutch. In the most extreme cases of reduced care, parents remain close to their eggs for only a few hours. Asterisks denote species for which there is experimental evidence that parental presence increases offspring survival. (b) Mean (± SE) time that females remain with their eggs after oviposition in *E. prosoblepon* (data from this study), *T. pulverata* and *C. granulosa* (Delia et al., 2017). (c) Mean (± SE) percentage of the embryonic development period that females remain with their eggs relative to the entire period of embryonic development across these three species (mean ± SE duration of embryonic development: *E. prosoblepon:* 25.44 ± 0.8 days, *N* = 16 (data obtained by J.G.V. in 2019); *T. pulverata*: 12.56 ± 0.36 days, *N* = 14 (Delia et al., 2019); *C. granulosa*: 12.1 ± 0.56 days, *N* = 4 (Delia et al., 2019)).

clutches before egg hatching (Fig. 1a). For instance, females of Hansen's Asian treefrog, *Feihyla hansenae*, stay with their eggs overnight and, at times, even during the day, throughout a significant portion of their embryonic development (Sheridan & Ocock, 2008). Likewise, females of the Magdalena giant glass frog, *Ikakogi tayrona*, remain with their clutches for only a few days after oviposition occurs (Bravo Valencia & Delia, 2016). In other species, such as banded-limb glass frogs, *Hyalinobatrachium cappellei* (Valencia-Aguilar et al., 2020), Eiffinger's treefrogs, *Kurixalus eiffingeri* (Chen et al., 2007), and rock frogs, *Thoropa taophora* (Consolgmano et al., 2016), males provide care to the eggs mostly during nocturnal periods. Finally, males of the Sri Lanka rock frog, *Nannophrys ceylonensis*, primarily attend their eggs during the day, leaving the clutch at night to forage (Wickramasinghe et al., 2004). Studies in which parent removal experiments were performed have shown that parental care increases egg survival even when parents temporarily abandon their clutches (Fig. 1a).

Most species of glass frogs (Centrolenidae) exhibit exclusive male egg attendance, in which males remain with their clutches for extended periods, often until egg hatching (Delia et al., 2020). Observational and experimental evidence from various species has consistently demonstrated that male presence increases egg survival, minimizing the risks associated with dehydration and predation (Delia et al., 2013, 2020; Lehtinen et al., 2014; Ospina-L et al., 2020; Salgado & Guayasamin, 2018; Valencia-Aguilar et al., 2020; Vockenhuber et al., 2009). Maternal care is comparatively less prevalent and has been experimentally demonstrated in only a few species of glass frogs (i.e. *I. tayrona*: Bravo Valencia & Delia, 2016; granular glass frog, *Cochranella granulosa*, and dusty glass frog, *Teratohyla pulverata*: Delia et al., 2017). In *C. granulosa* and *T. pulverata,* females exhibit short-term parental care, remaining with their eggs for a maximum of 3 h (Delia et al., 2017; Fig. 1b, c). Female removal experiments have revealed that, despite the brevity of this period of egg attendance, it significantly decreases the risks of dehydration and, to a lesser extent, predation when compared to clutches in which females are absent.

Females of the emerald glass frog, *Espadarana prosoblepon*, remain with their eggs for no more than 2 h after oviposition (Goyes Vallejos & Hernández-Figueroa, 2022), which represents, on average, nearly half of the time reported for *T. pulverata* and *C. granulosa* (Fig. 1b, c). Considering that the temporal association between an *E. prosoblepon* female and her clutch accounts for only 0.15% of the total duration of embryonic development (Fig. 1c), we aimed to investigate whether this short-term female presence close to the eggs could increase their fitness. If the brief period during which females remain with their clutches after oviposition has the same function as the short-term care provided by *T. pulverata* and *C. granulosa*, we hypothesize that female presence in *E. prosoblepon* enhances overall egg survival. If so, female presence should reduce egg mortality caused by dehydration and predation compared to clutches in which females are removed immediately after oviposition. The results obtained from this study elucidate whether even an exceedingly brief period of female presence confers benefits to the offspring, thereby providing insights into the required duration of parental presence to characterize parental care behaviour.

METHODS

Study Area

We studied a population of *E. prosoblepon* found at Las Cruces Biological Station, Coto Brus County, southern Puntarenas Province, Costa Rica (8°47′10″N, 82°57′32″W, World Geodetic System datum = WGS84, 1100 m above sea level). Our study site has an area of approximately 600 m^2 crossed by a ditch system within the station's Botanical Garden surrounding a 60 m long segment of Culvert Creek. Fieldwork took place between June and July 2021 and 2022, corresponding to the beginning of the rainy season (May–November).

Study System

Males of *E. prosoblepon* call for mate attraction on top of leaves and branches throughout our study site. After engaging in amplexus, females lay eggs on various oviposition sites on the banks of Culvert Creek (Fig. 2). The height at which clutches are deposited ranges from 0 to 340 cm (mean ± SD = 84.7 ± 68.5 cm, $N = 48$). Most females laid their eggs on horizontally creeping spike moss, *Selaginella diffusa* (31.2%), on patches of leafy liverworts and mosses growing on tree trunks, rocks and stream banks (25%) and on leaf litter (20.8%) (Fig. 2). Only 12.5% of the females deposited their eggs on the upper side of overhanging leaves of various plant species, including fern blades and Kunze club moss, *Selaginella haematodes*. Females also laid eggs on assorted plant surfaces, such as the petiole and spicules of the giant fern *Angiopteris evecta* and other plant stems, as well as on muddy banks (Fig. 2). Temperature and relative humidity at the microhabitat where the eggs were laid ranged from 17.0 to 25.7 °C (mean ± SD = 19.1 ± 1.7 °C) and from 84% to 100% (97.1 ± 4.5%), respectively.

Effect of Female Presence on Clutch Hydration

We conducted a female removal experiment in semicaptivity to test whether females provide any supplemental hydration after oviposition. To evaluate the level of clutch hydration, we developed a novel method that allowed us to quantify the thickness of the gelatinous coat around the eggs. We used the nearest-neighbour distance among eggs in each clutch to estimate the gelatinous thickness around the eggs. When the clutch dehydrates, the gelatinous coat around the eggs shrinks, causing the eggs to be closer to each other compared to hydrated clutches in which the gelatinous coat increases the distance between eggs (Fig. 3a, b).

Using nearest-neighbour distances, we evaluated whether clutches in which the females were allowed to remain with their eggs (control group) had values of gelatinous thickness different from clutches in which females were experimentally removed (removal group). We predicted that, if the female provided supplemental hydration to the clutch, then the nearest-neighbour distance values would be greater in clutches from the control group compared to clutches from the removal group.

Between 14 June and 16 July 2022, we searched for pairs of *E. prosoblepon* in amplexus every night, beginning at 2000 hours until approximately 2300 hours. Pairs were found at a height of 12–144 cm (mean ± SD = 59.8 ± 36.1 cm). Once we found a pair in amplexus, we transferred it to an individual fine-mesh outdoor enclosure (base: 40 × 30 cm; height: 40 cm) with a clear vinyl window, which allowed us to observe the behaviour of the individuals inside (Fig. 3c). Each enclosure had an oviposition substrate (a fern frond), leaf litter at the bottom and water. The enclosures were located at our study site to replicate the environmental conditions experienced by mating pairs in the wild. After transferring two pairs to their respective enclosures, we randomly assigned them to the control or removal group. We monitored the pairs under dim red light from 0000 to 0600 hours through continuous observation.

We had 22 successful trials in both experimental groups. Every control–removal dyad happened on the same day (except in four cases) to account for differences in temperature, humidity and rainfall. For the control group, we recorded how long the female remained with the clutch. For the removal group, we removed the

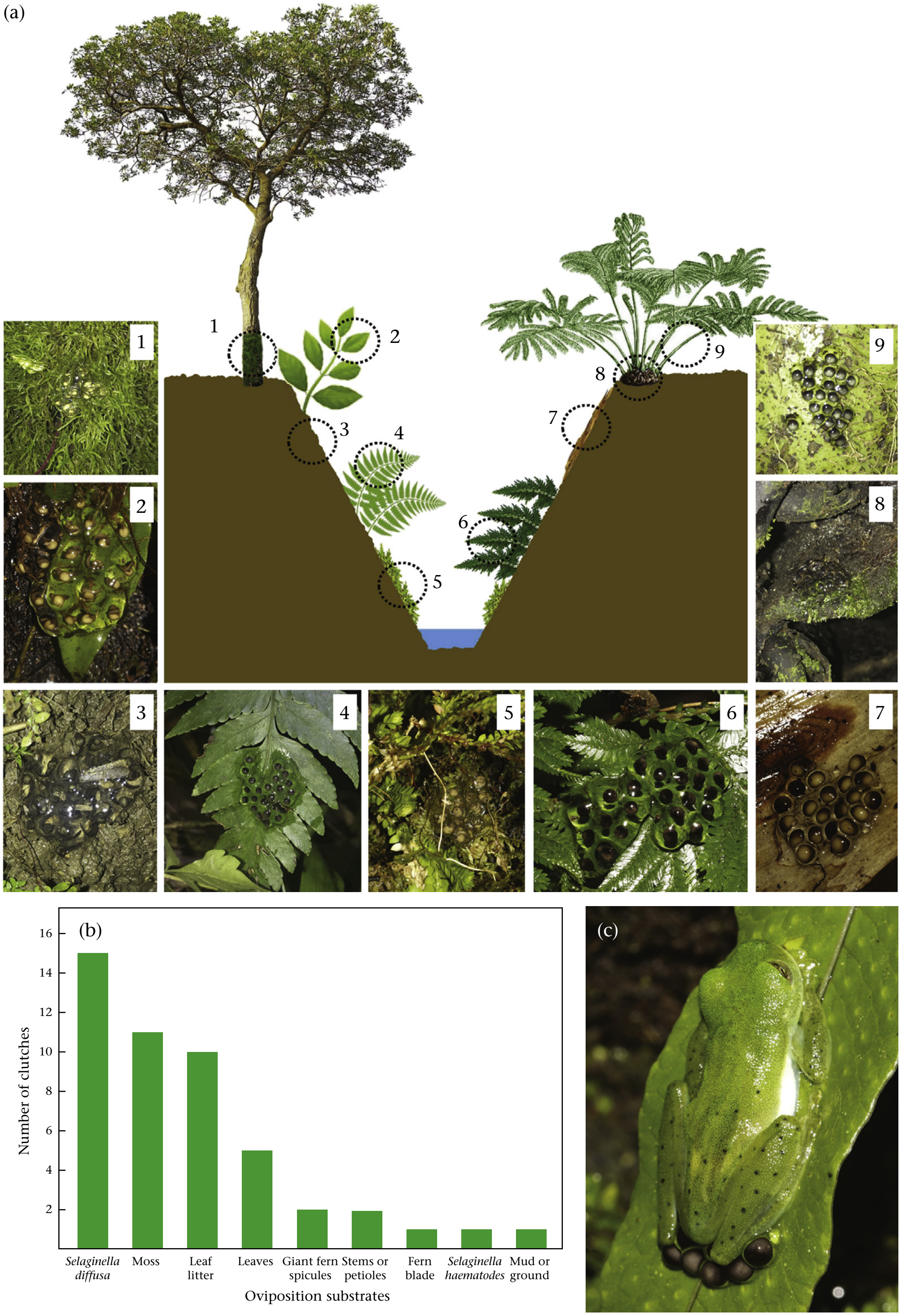

Figure 2. (a) Transversal view of Culvert Creek showing the substrates used as oviposition sites by female emerald glass frogs: (1) moss, (2) leaves, (3) mud, (4) fern blades, (5) *S. diffusa*, (6) S. haematodes, (7) leaf litter, (8) spicules of the giant fern *A. evecta* and (9) stems or petioles. For better visualization, these substrates are not to scale. (b) Number of clutches found in each oviposition substrate throughout the study period ($N = 48$). After oviposition, females remained close to their eggs for up to 113 min (c).

female immediately after oviposition by prodding her gently with a paintbrush. The following night (ca. 1900 hours), we individually marked the females with Visible Implant Alpha tags (Northwest Marine Technology, Olympia, WA, U.S.A.) to avoid capturing the same female twice throughout the study period. Pairs were then released at the point of capture within 24 h.

To determine whether the estimates of nearest-neighbour distance differed between the clutches of the two experimental groups, for the control group, we took one photo of the clutch on a 90° flat surface immediately after the female left the egg clutch. For the removal group, we took one photograph of the clutch immediately after the removal of the female ($t_0 = 0$ min), and another

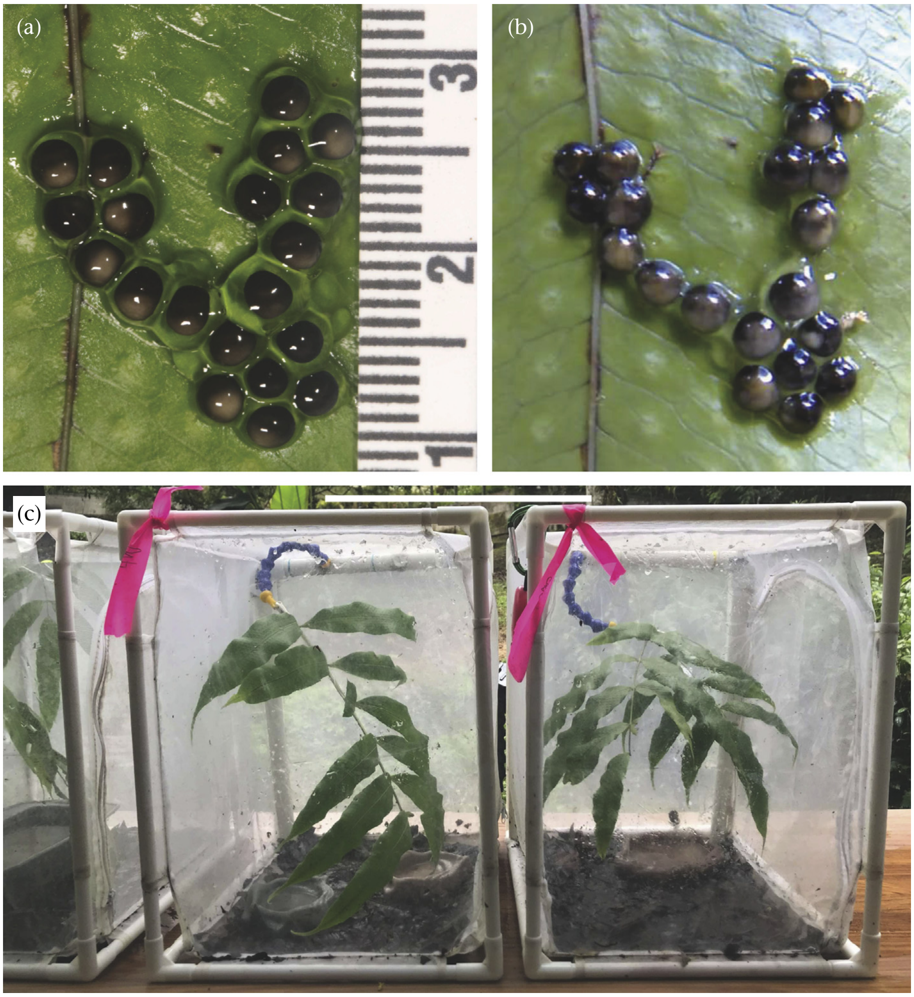

Figure 3. (a–b) Photos of the same clutch of an emerald glass frog showing how the level of hydration of the gelatinous coat changes the distance between eggs. In (a), the clutch was photographed 25 min after the female deserted it (0800 hours) and the gelatinous coat around the eggs is thick. In this situation, eggs are far apart and the value of the nearest neighbour distance is 1.87 mm. In (b), the clutch was photographed 10 h after the first photograph in (a) and the gelatinous coat around the eggs is dehydrated and thin. In this situation, eggs are close to each other and the value of nearest-neighbour distance drops to 1.18 mm. (c) Enclosure where we conducted the experiment to evaluate the effect of female presence on clutch hydration. Each enclosure (one for each experimental group; female present versus female removed) contained a fern frond to be used as oviposition site, leaf litter on the bottom and a bowl containing water.

photograph at 90 min (t_{90}), which is the average (+1 SD) time that females remain with the clutch (Goyes Vallejos & Hernández-Figueroa, 2022). We emphasize that taking a photograph at t_0 for females in the control group was not feasible due to females being susceptible to abandoning their clutches in response to even minimal disturbance (see Results).

We quantified the nearest-neighbour distance for each egg within a clutch using the ‘Nnd’ plugin in the open-source image processing software FIJI (Schneider et al., 2012). The ‘Nnd’ plugin numerically identified and labelled each egg in the photographs. Subsequently, the plugin calculated the distance of each egg to its nearest neighbour (shortest distance), thus ensuring a single nearest-neighbour distance measurement for each of them (see Appendix, Fig. A1). To avoid redundancy in the analysis, instances where the nearest neighbour for egg *i* was *j*, and vice versa, were identified and consolidated into a single entry. This procedure ensured that each pair of closest eggs contributed only one distance measurement in the analysis.

We analysed the effect of the experimental group (control versus removal t_0 and removal t_{90}) on the nearest-neighbour distance estimates fitting a linear mixed-effects model using the ‘lmer’ function in the package ‘lme4’ (Bates et al., 2015). The model summary and results were examined using the ‘Anova’ function in the ‘lmerTest’ package (Kuznetsova et al., 2017). We performed post hoc pairwise comparisons using the ‘glht’ function in the ‘multcomp’ package (Hothorn et al., 2008) to identify differences between the experimental groups. Additionally, we conducted a paired analysis, comparing the nearest-neighbour distance estimates within the removal group at t_0 and t_{90} using a linear mixed-effects model with time as a fixed factor. These models incorporated clutch identity (ID) as a random effect to account for (1) multiple nearest-neighbour distance measures within the same clutch, (2) within-clutch variance in nearest-neighbour distances and (3) clutch repeatability (between removal t_0 and t_{90}). Specifically, these analyses enabled us to examine whether maternal presence enhances clutch hydration and whether clutches dehydrate over time without a mother.

Maternal Commitment Assay

We simulated predation risk by using a paintbrush as a disturbance stimulus to explore females' commitment to remain with their eggs (modified from Delia et al., 2017). Given that some individuals of *E. prosoblepon* exhibit a kicking behaviour when gently brushed (J. Goyes Vallejos, personal observation), we wanted to assess whether females would behaviourally defend their clutches when experimentally disturbed. Moreover, we wanted to assess the magnitude of simulated predation risk that they would tolerate before abandoning the clutch. We followed 14 pairs in amplexus and waited until oviposition occurred and the male had left the oviposition site. Then, we gently brushed the female's leg with the paintbrush (predation risk stimulus) and waited 15 s for a response before disturbing her again. We used infrared video cameras to record any kicking or other defensive behaviours and to determine the number of stimuli needed for the female to flee. After female desertion occurred, we continued video recording the oviposition site until dawn (ca. 10 h, except in three instances in which the cameras stopped recording after 1 h) to determine whether females returned to their clutches. The return to the clutch could be interpreted as another indication of maternal commitment (Delia et al., 2017).

Female Removal Experiment in the Field

We conducted a female removal experiment in the field to determine the function of the short-term association between females and their clutches. To do this, we searched for pairs in amplexus throughout the study area from 8 June to 11 July 2021, starting at 2000 hours. Pairs in amplexus were found between 2000 hours and 0200 hours. Once a pair was located, we monitored its behaviour using a dim red light to minimize disturbance of the individuals during observation. The first pair on a given sampling night was randomly assigned to the removal or control group; the next pair was assigned to the opposite experimental group. This was done to have both experimental groups evenly allocated across time. Most pairs in amplexus travelled 0.2–5.7 m from the point where they were first observed (mean ± SD = 2.4 ± 1.5 m). Pairs were followed through the vegetation until oviposition occurred. Oviposition usually took place between 2300 hours and 0500 hours the next day, with the majority occurring between 0000 hours and 0300 hours ($N = 46$).

Females assigned to the removal group were removed from their clutches immediately after oviposition. Females assigned to the control group were allowed to remain with their clutches until they deserted them and started wandering on the vegetation. After removal or voluntary desertion, females were individually marked with Visible Implant Alpha Tags (Northwest Marine Technology) to avoid capturing the same female twice throughout the study period. After the marking procedure, we released the females at the same point where they were captured. The time at which oviposition occurred and the total number of eggs per clutch were recorded for females of both experimental groups.

For the control group, we recorded how long the female remained with the clutch (Fig. 1b, c). Egg survival, calculated as the number of eggs surviving until hatching, was recorded for the control and removal groups. We monitored clutches daily and recorded the number of hatched tadpoles using a sealed plastic funnel positioned and secured under each of the clutches. At the onset of hatching, tadpoles were counted every day and released immediately onto the stream. Concurrently, we identified specific sources of egg mortality: (1) failure to develop, characterized by embryos ceasing development before or at Gosner stage 18 (Gosner, 1960); (2) fungal infection, characterized by cloudy eggs with visible hyphae on the surface; (3) dehydration, characterized by underdeveloped eggs lacking jelly around them; (4) drowning or rain-stripping, where entire clutches disappeared after heavy precipitation; (5) predation, characterized by the disappearance of eggs or late-stage embryos, particularly around the edge of the clutch, leaving some embryos in the centre, or characterized by empty gelatinous masses before hatching started (sensu Hawley, 2006; Warkentin, 2000).

We fitted a generalized linear model (GLM) with beta-binomial distribution and logit link function using the ‘glm’ function in R to determine whether the overall proportion of tadpoles hatched was influenced by the experimental group. We also fitted an intercept-only model with the proportion of hatched tadpoles as a response variable. We used a likelihood ratio test (LRT) of significance for model comparison. Finally, we compared control and removal groups for each source of mortality separately using a GLM with beta-binomial distribution and cloglog link function.

Unless otherwise stated, we present summary statistics as means ± SD. All statistical analyses were conducted in R version 4.2.0 (R Core Team, 2023). Figure plots were generated in R with the ‘ggplot2’ package (Wickham et al., 2016). Schematic representations and diagrams were done with Microsoft Power Point version 16.82 (Microsoft Corp, Redmont, WA, U.S.A.).

Ethical Note

The tags for individual identification measured 1.0 × 2.5 mm and were inserted just below the skin on the left thigh of each frog. The process involved a disinfected injector immersed in 90% ethanol before each marking procedure and between individuals, ensuring a hygienic process. The entire procedure lasted less than 5 min per individual and was conducted with the utmost care to minimize stress on the frogs. Following tagging, individuals were gently sprayed with water and their behaviour was observed to detect any anomalies. Importantly, field observations have indicated that individuals behave normally throughout the field season after tagging and even across multiple years. Moreover, Visible Implant Alpha tags have been widely used and have not been demonstrated to negatively impact amphibians (Heard et al., 2008; Knapp et al., 2023). Given that the tags are visible without manipulation, even while individuals are in amplexus, we could avoid unnecessary recaptures.

During experiments in semicaptivity, when an amplectant pair was found, we placed the pair inside a plastic bag, ensuring careful handling to avoid dislodging the male, and then transferred them to an outdoor enclosure within the field site. The entire capture and transfer procedure took less than 10 min due to the strategic location of the enclosure within our study site. The plastic bag was placed inside the enclosure and the pair was gently prodded to leave the bag. The pairs remained in the enclosure for no more than 24 h and, during this period, exhibited behaviour similar to that observed in the field. Clutches obtained in the semicaptivity experiments were transferred (still attached to the provided substrate) to their natural habitat to continue their embryonic development. In turn, clutches used in the removal experiment in the field were monitored until the onset of hatching and the tadpoles were released into the creek below the oviposition site.

All behavioural observations and field manipulations followed the ASAB/ABS Guidelines for the treatment of animals in behavioural research and teaching. Our study was approved by the Costa Rican Ministry of the Environment and Energy (MINAE) and the National System of Conservation Areas (SINAC) (approval numbers: R-SINAC-PNI-ACLAP-036-2021 and M-P-SINAC-PNI-ACAT-007-2022), as well as the Animal Care and Use Committee (ACUC) at the University of Missouri (Protocol No. 10164).

RESULTS

Female Removal Experiment in Semicaptivity

Females in the control group remained with their clutches for an average of 53.1 ± 22.8 min after oviposition (range 25–86 min, $N = 7$). For four pairs, we were not able to calculate how long the female remained with the clutch because we did not observe the precise moment when oviposition occurred. However, we were able to observe the females until they deserted their clutches. There was no difference in clutch size between the two experimental groups (t test: $t_{20} = 0.474$, $P = 0.641$; control = 27.45 ± 6.61 eggs, $N = 11$; removal = 26.27 ± 4.98 eggs, $N = 11$).

The average nearest-neighbour distance was 1.83 ± 0.18 mm for clutches from the control group and 1.78 ± 0.13 mm and 1.81 ± 0.16 mm, respectively, for clutches from the removal group at 0 min (t_0) and 90 min (t_{90}) (Fig. 4). The statistical analysis showed that the variable ‘experimental group’ had a significant effect ($\chi^2_2 = 8.313$, $P = 0.015$). Upon comparison among experimental groups, we found that there was no significant difference in nearest-neighbour distances between the control and the removal group at t_0 ($P = 0.666$) or between the control and the removal group at t_{90} ($P = 0.945$). However, there was a significant difference in the nearest-neighbour distance between removal t_0 and removal t_{90} (coefficient = 0.034, SE = 0.012, $t = 3.130$, $P = 0.002$). Removal clutches tended to increase the distance between eggs after 90 min had elapsed (Fig. 4).

Maternal Commitment Assay

After oviposition, most of the females ($N = 11$ out of 14, 78.6%) fled after one to four touches with the brush, with six out of 11 females jumping off the oviposition substrate before initiating stimulus or after the first touch. Only three of 14 females required repeated stimuli to induce abandonment of the clutch: one at 4 min, one at 7 min and one at 25 min. None of the 14 females returned to their clutch after abandonment.

Female Removal Experiment in the Field

In the control group, females stayed with their clutches for 54.6 ± 26.0 min (range 16–113 min; $N = 23$), with 17 females (74% of the total) remaining within the time range of 34–72 min. There was no difference in clutch size between the two experimental groups (t test: $t_{48} = 0.221$, $P = 0.834$; control = 22.7 ± 4.39 eggs, $N = 23$; removal = 22.5 ± 3.88 eggs, $N = 25$). The mean percentage of total hatching success was 50.7 ± 39.8% for clutches from the control group and 55.8 ± 38.0% for clutches from the removal group. The model comparison indicated that incorporating the variable ‘experimental group’ did not enhance the model compared to the intercept-only model. These results suggest no significant differences in hatching success between the experimental groups ($\chi^2_1 = 0.002$, $P = 0.968$; Fig. 5).

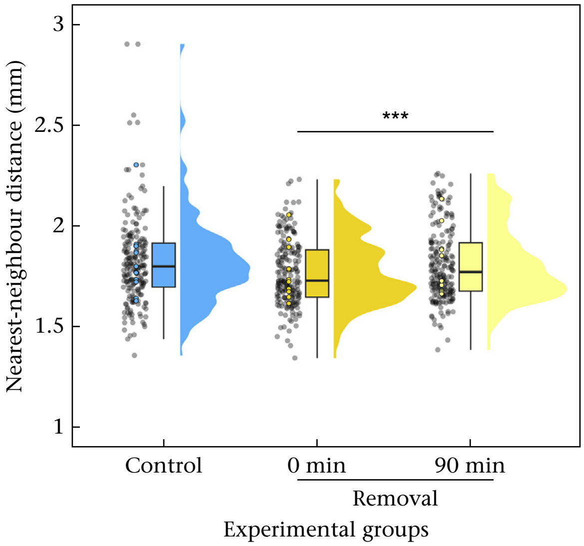

Figure 4. Raincloud plot comparing the average nearest-neighbour distance between the eggs from two experimental groups: control and removal. The measurements for the removal group were taken at 0 min (t_0) and 90 min (t_{90}). The grey dots represent the raw nearest-neighbour distance values for all clutches within each experimental group, followed by a box plot and the probability density of the data at different values. The colour dots represent the mean nearest-neighbour distance per clutch (control: $N = 11$, removal: $N = 11$). ***$P = 0.002$.

Predation by invertebrates was the most common source of egg mortality in both experimental groups (proportion of eggs consumed: control = 31.1 ± 39.7%; removal = 20.7 ± 37.3), but there was no significant difference between them ($\chi^2_1 = 0.789$, $P = 0.375$; Fig. 5). Other causes of egg mortality included failure to develop, fungal infection, dehydration and rain-stripping (Fig. 5). We included an ‘unknown’ category for cases in which eggs or complete clutches disappeared and it was unclear whether it was due to a predation or rain-stripping event. There was no difference between experimental groups in the proportion of egg mortality caused by failure to develop (control = 4.8 ± 7.2%, removal = 9.9 ± 12.9%; $\chi^2_1 = 0.998$, $P = 0.319$) and unknown causes (control = 4.8 ± 13.3%, removal = 9.3 ± 18.0; $\chi^2_1 = 0.773$, $P = 0.380$). We did not perform statistical comparisons between the experimental groups for three of the sources of egg mortality, namely dehydration ($N = 1$ in the control group), fungal infection ($N = 2$ in the removal group) and rain-stripping ($N = 2$ in each experimental group) because sample sizes were small and represented only a minor proportion of the total egg mortality (Fig. 5).

DISCUSSION

In this study, we investigated whether the brief presence of females of the emerald glass frog close to their clutches, which accounts for only 0.15% of the total duration of embryonic development (Fig. 1c), increases egg survival. The removal experiment in semicaptivity provided no evidence that female presence improves egg hydration after oviposition. In the maternal commitment assay, most females subjected to a gentle disturbance abandoned their clutches within the first minute after oviposition and did not return to their clutches. Lastly, clutches with or without mothers experienced similar levels of mortality, with both experimental groups facing predation by invertebrates as the primary cause of egg mortality. Collectively, these results clearly indicate that the brief presence of females close to their clutches does not confer any measurable fitness benefit to the eggs.

In terrestrially breeding amphibians, the risk of clutch dehydration exerts a strong selective pressure on egg survival, potentially driving the evolution of egg attendance in some species (Wells, 2007; Vági et al., 2019). In the glass frogs *C. granulosa* and *T. pulverata*, even a short-term female permanence with their eggs increases clutch hydration four-fold compared to clutches without females (Delia et al., 2017). This suggests that females can provide water to their eggs through close contact with their ventral pouch (osmosis) or by urinating over them, as has been reported for other frogs and salamanders (e.g. Forester, 1984; Poo & Bickford, 2013). However, in our female removal experiment in semicaptivity, the short-term permanence of females did not affect egg hydration since clutches with and without females maintained similar hydration levels even after the average duration of female presence with the clutches had elapsed. Surprisingly, clutches from the removal group exhibited a significant increase in the distance between the eggs after 90 min. The noticeable thickening of the gelatinous egg casings indicates that the clutches do not dehydrate over time without the female. Instead, it implies that the eggs may absorb moisture from the high-humidity environment. Moreover,

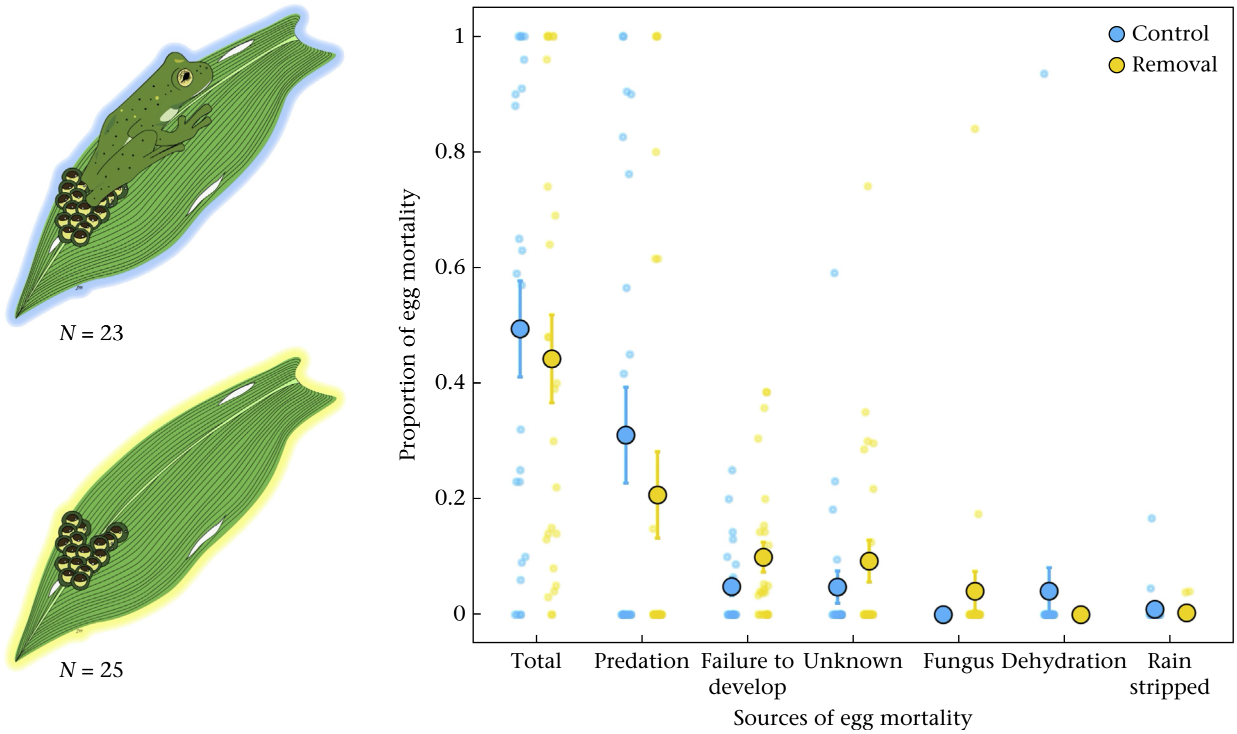

Figure 5. Mean (± SE) proportion of the total egg mortality and specific sources of mortality observed in a female removal experiment in the field for the control and removal groups. The specific sources of egg mortality for the control and removal groups were divided into six categories.

in our field experiment, only one clutch suffered dehydration, suggesting that this is not a relevant cause of egg mortality in *E. prosoblepon*. In fact, a previous study demonstrated that the primary sources of egg mortality in clutches experimentally placed on overhanging vegetation are predation and rain-stripping rather than dehydration (Goyes Vallejos & Ramirez-Soto, 2020). Our study site experiences nearly continuous rainfall from May to October, which likely reduces the risk of dehydration due to regular downpours. Constant rainfall may also explain the wide diversity of substrates used by *E. prosoblepon* females as oviposition sites, contrasting with the typical pattern observed in other glass frogs that primarily lay eggs on the upper or underside of leaves overhanging small streams (reviewed in Delia et al., 2020). Thus, even though females do not increase egg survival by remaining close to their clutches for a brief time, they could still protect their offspring by selecting hidden oviposition sites, such as patches of *S. diffusa*, moss, leaf litter and spicules of giant ferns, which together account for nearly 80% of the oviposition sites (Fig. 2). In these sites, the risk of rain-stripping is likely lower, and the concealed eggs are probably protected from some types of predators, especially those visually oriented. Future research should explore how abiotic variables (i.e. precipitation, temperature, humidity) at the microhabitat level and the choice of oviposition substrate may influence egg survival.

Parental removal experiments conducted on several frog species showing exclusive male care have demonstrated the crucial role of egg attendance in increasing egg survival (Machado & Macedo-Rego, 2023). However, removal experiments involving species with exclusive female care are comparatively less common and have only been conducted on a few species. In *F. hansenae*, for instance, females consistently stay near their clutches, and the maternal presence increases egg survival by preventing dehydration and predation (Poo & Bickford, 2013). In glass frogs, females of *C. cochranella* and *T. pulverata* remain close to their clutches for less than 3 h after oviposition (Fig. 1b), and, despite this relatively short attendance period, maternal presence reduces egg mortality due to dehydration in both species and reduces predation in *T. pulverata* (Delia et al., 2017). Contrastingly, *E. prosoblepon* females remain close to their clutches for no more than 1.5 h (Fig. 1b), and our results reveal that maternal presence does not improve clutch hydration or decrease egg mortality due to predation. Comparing the time females remain close to their eggs in our study species to that reported for *C. granulosa* and *T. pulverata*, we find that the embryonic development period in *E. prosoblepon* is twice as long as that of the other two glass frog species. Consequently, the proportion of time *E. prosoblepon* females spend with their clutches is negligible when compared to those two species (Fig. 1c). Considering that this short-term maternal presence has no clear benefit to the eggs, this female behaviour does not meet the definition of parental care, which implies a parental behaviour that increases offspring fitness (sensu Smiseth et al., 2012).

In frog species with parental care, parents often engage in defensive behaviours that protect their offspring from predators (Townsend et al., 1984). For instance, in Savage's glass frogs, *Centrolene savagei*, and reticulated glass frogs, *Hyalinobatrachium valerioi*, two species with prolonged male egg attendance, males actively deter predatory wasps and ants by lunging at them or kicking them (Ospina-L et al., 2020; Vockenhuber et al., 2009). Analogous defensive behaviours against potential egg predators have also been observed in other taxa that exhibit egg attendance, including insects (Kudô et al., 1989; Tallamy & Denno, 1981), arachnids (e.g. Requena et al., 2009; Willemart & Gnaspini, 2004) and salamanders (Forester, 1979). Antipredator behaviours are closely linked to the parent's commitment to defending their eggs (Figler et al., 1995). In the case of *C. savagei*, males attending eggs exhibit kicking and biting behaviours when disturbed by a brush (a predation stimulus), whereas nonattending males flee within 40 s of the stimulus onset (Ospina-L et al., 2020). Similarly, in the glass frog *I. tayrona*, a species with exclusive maternal care, females attending eggs resist intense physical stimuli, suggesting a high level of commitment to offspring protection (Bravo Valencia & Delia, 2016). However, *E. prosoblepon* females with recently laid clutches do not display defensive behaviours, and most of them flee immediately after a gentle disturbance. This finding indicates they behave similarly to nonattending individuals of *C. savagei* and *I. tayrona* (Bravo Valencia & Delia, 2016; Ospina-L et al., 2020). The absence of defensive behaviours and the immediate flight response observed in our study species reinforce the notion that the short-term female permanence close to their eggs cannot be considered parental care. But if *E. prosoblepon* females are not protecting the offspring against dehydration or predators, a question arises: why do they remain with the eggs for a brief period after oviposition?

After amplexus, *E. prosoblepon* females travel, on average, 2.4 m while carrying the male on their backs. Long-term studies with 13 terrestrial frog species from six families show that the mean (± SD) distance moved during routine activities is 9.16 ± 9.07 m (values calculated from median distance values between captures; Table 6.3 in Wells, 2007). Thus, the mean displacement of amplectant females in a single night is nearly a quarter of the mean distance per move reported for other frog species across days. Given that males represent about 78% of the females' total mass (Goyes Vallejos & Hernández-Figueroa, 2022), prolonged movement during amplexus likely results in high energy expenditure for females. A study on cane toads, *Rhinella marina*, showed that the extended amplexus reduces female sprint and swim performance, with the extent of locomotor impairment depending on the mass of the amplectant male during terrestrial locomotion trials (Bowcock et al., 2009). Although not explored by the authors, reduced locomotor performance linked to the load carried by females probably increases energy expenditure. To our knowledge, there is only one study that quantified the metabolic cost of amplexus in females. The author did not detect a significant effect of amplexus on female metabolic rate in the grey treefrog, *Hyla versicolor* (McLister, 2003), but the measurements were conducted in the laboratory with stationary pairs in amplexus. Assuming that carrying a male increases the females' energy expenditure in *E. prosoblepon*, the short-term permanence close to their eggs after oviposition may represent a recovery period during which females remain quiescent. In fact, the posture of females after oviposition (Fig. 2) resembles sleeping individuals of other frog species, with limbs tucked under their bodies, head down and eyes covered with the nictitating membrane (Hobson et al., 1968). Future studies could explore the potential correlations between the female quiescence period and male size or the male-to-female ratio during amplexus. Investigating the interplay between these variables and other factors, such as amplexus duration or distance travelled searching for oviposition sites, could provide insights into the energetic costs that females incur and how it could affect their postoviposition quiescence behaviour.

Our study's main conclusion is that the short-term period *E. prosoblepon* females stay with their clutches after oviposition does not confer a benefit in the form of increased egg survival and, therefore, cannot be regarded as parental care. This finding contrasts with two other previously studied glass frog species, namely *C. granulosa* and *T. pulverata*, in which a slightly longer permanence of the females close to their clutches after oviposition reduces egg dehydration and predation (Delia et al., 2017). While the time *E. prosoblepon* females remain with their clutches is not enough to confer a demonstrable benefit to the eggs, we suggest that females could increase egg survival based on oviposition site selection, which is perhaps the most widespread form of preovipositional parental care in animals (Smiseth et al., 2012). However, to be considered parental care, oviposition site selection must increase offspring fitness, providing suitable conditions for egg development and survival. Thus, the next step is to investigate whether the selection of substrates for oviposition reduces egg mortality. Lastly, our findings challenge the assumption that the proximity of parents and offspring indicates parental care behaviour. We caution against assuming parental care solely based on adult presence near the offspring and encourage researchers to thoroughly test assumptions when reporting natural history observations related to parental care.

Author Contributions

J.G.V. conceived the study and methodology, contributed to data collection, conducted the experiments, analysed the data, prepared figures and drafted, wrote, reviewed and edited the manuscript. **J.S.S.**, **V.C.** and **N.R.** contributed to data collection, conducted experiments and reviewed drafts of the manuscript. **G.M.** prepared figures and wrote, reviewed and edited the manuscript.

Data Availability

All data supporting the findings of this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.612jm648q (Goyes Vallejos et al., 2024).

Declaration of Interest

The authors declare no competing interests.

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Appendix

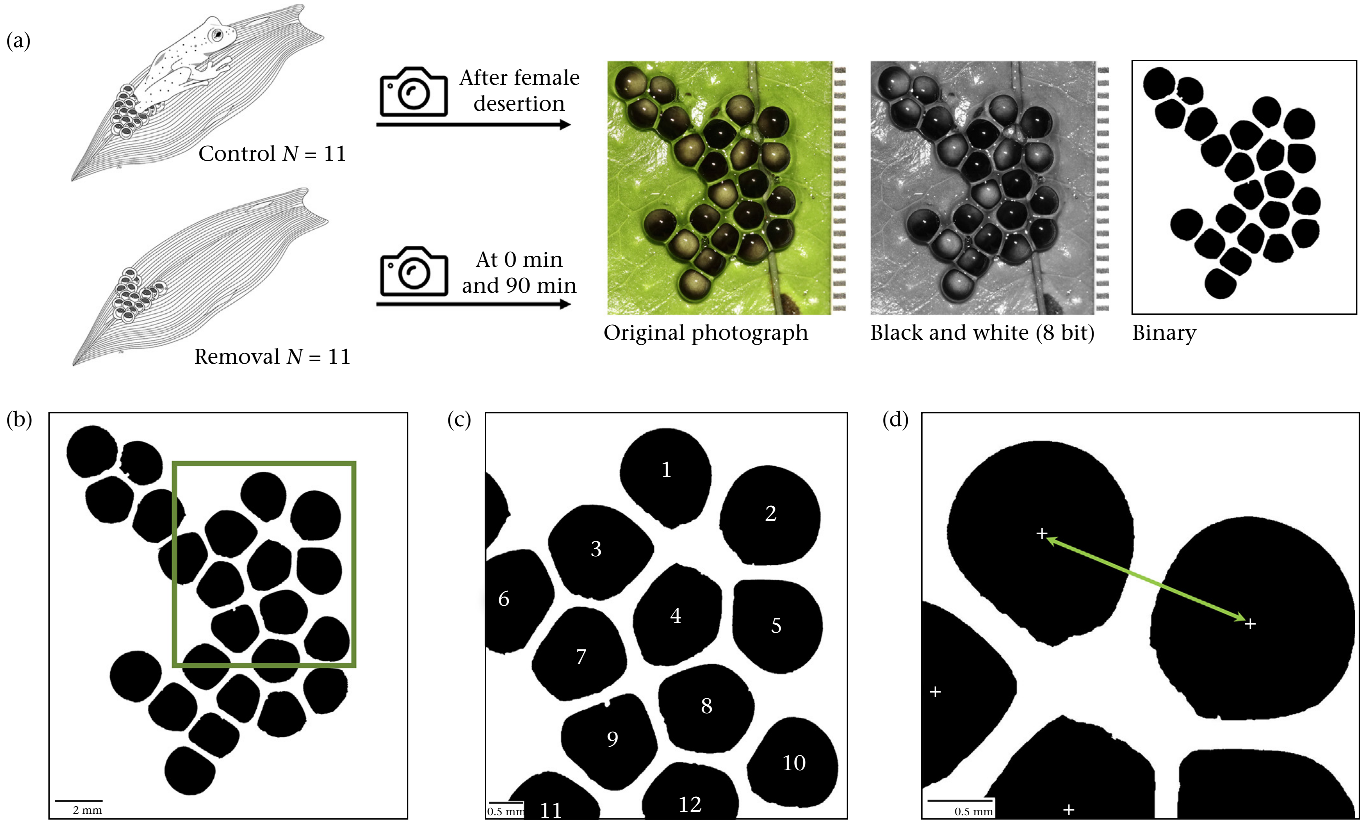

Figure A1. Method employed for estimating the nearest-neighbour distance. (a) In the control treatment, a single photograph of the clutch on a 90° flat surface was taken immediately after the female left the egg clutch. For the removal treatment, two photographs were taken: one right after the female's removal (0 min) and another at 90 min. The clutches' average nearest-neighbour distance was calculated using FIJI image processing software. The photographs were processed into black and white (8-bit) and subsequently converted into binary format. (b) The ‘Nnd’ plugin in FIJI identifies all particles (in this case, eggs) and (c) assigns a unique identifier number to each particle. (d) The plugin then calculates the centre for each particle (denoted with a + symbol) and computes the nearest-neighbour distance between two particles from their centres (green arrow), representing the shortest distance between two eggs.