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NOTE

Larvae of the blow fly *Caiusa testacea* (Diptera: Calliphoridae) as egg predators of *Polypedates cruciger* Blyth, 1852 (Amphibia: Anura: Rhacophoridae)

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Habitat destruction and alteration have been identified as the most detrimental causes of amphibian decline (Kiesecker 2003). The effects of climate change and amphibian diseases, however, are emerging topics, and have taken increased attention in conservation approaches regarding the amphibian fauna (Hayes et al. 2010; Li et al. 2013). Predatory pressure during different life stages of amphibians is another factor that significantly affects populations (Chivers et al. 2001; Blaustein et al. 2012). Diverse invertebrate and vertebrate fauna prey on eggs and tadpoles of aquatic and terrestrial nesting anurans (De Silva 2001a,b; Lingnau & Di-Bernardo 2006). According to Downie (1990), terrestrial foam nests of Rhacophoridae have evolved to protect eggs and embryos from aquatic predators. Some vertebrates (e.g., monkeys and snakes) and invertebrates (e.g., beetles, ephydrid flies, phorid flies, spiders, ants, and blow flies), however, have been identified as egg predators of anuran foam nests (Vonesh 2000; Rödel et al. 2002; Menin & Giaretta 2003; Lingnau & Di-Bernardo 2006; Banerjee et al. 2018). Blow flies of the genus *Caiusa* (Diptera: Calliphoridae) are one of the major predators of terrestrial Rhacophoridae eggs (Rognes 2015). These flies are one of the major reasons for embryo mortality of some rhacophorid genera, including *Chiromantis*, *Feihyla*, *Polypedates*, and *Rhacophorus* (Lin & Lue 2000). So far, seven known species of *Caiusa* (*C. borneoensis* Rognes, 2015, *C. coomani* Séguy, 1948, *C. indica* Surcouf, 1920, *C. karrakerae* Rognes, 2015, *C. kurahashii* Rognes, 2015, *C. violacea* Séguy, 1925, and *C. pooae* Rognes, 2015) have been identified as foam nest predators and predators of jelly-like egg masses of anurans (Lin & Lue 2000; Rognes 2015; Banerjee et al. 2018). The emerging larvae of these fly species consume eggs and developing embryos in egg masses. There are knowledge gaps in our understanding of the fly-anuran interactions and the wider impact of these flies on anuran population dynamics.

Sri Lanka is a tropical country with more than 120 species of anurans, nearly 104 of which are endemic to the country (De Silva & Wijayathilaka 2019). Approximately 83 (69%) of the reported species belong to the family Rhacophoridae, including arboreal foam nesting *Polypedates* and *Taruga* species (Meegaskumbura et al.

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2010). The majority (more than 75%) of anuran species in the country are categorized as threatened, mainly due to anthropogenic activities (Manamendra-Arachchi & Meegaskumbura 2012; De Silva & Wijayathilaka 2019). Current conservation approaches are mainly aimed at minimizing habitat destruction and other adverse human activities. Only a few studies, however, have reported the effect of amphibian diseases on the population structure of anurans in Sri Lanka (De Silva 1999; Rajakaruna et al. 2007; Jayawardena et al. 2010; De Silva 2011), and hardly any studies have investigated predatory pressure on different life stages of amphibians in the country. Morgan-Davies (1958) reported *Caiusa indica* as predatory in foam nests of *Polypedates cruciger* Blyth, 1852 (Anura: Rhacophoridae) in Sri Lanka. According to De Silva & De Silva (2000), a species of Calliphoridae fly acts as an egg predator of *P. cruciger* frogs, however, these authors did not provide a species-level identification for the flies. Therefore, there are some literature gaps in information about predatory flies and their pressure on the developmental stages of anurans in Sri Lanka. Thus, detailed investigations including systematic and quantified studies to assess the damage caused by the egg predators to anuran eggs are important in relation to conservation actions. In this study, we identified natural dipteran predators of foam nests of *P. cruciger*, an endemic Rhacophoridae species in Sri Lanka. Further, we quantified the egg mortality of *P. cruciger* due to the infestation of the predatory dipteran fly.

The study was conducted from May 2019 to August 2019, at two localities [Gampola (7.150^{0}N, 80.555^{0}E) and Peradeniya (7.259^{0}N, 80.597^{0}E)] in the Kandy District of Sri Lanka. Spawns were searched for in microhabitats with *P. cruciger* (i.e., man-made ponds, cement water tanks, domestic wells, tree-holes, and organically managed agricultural lands). When a fresh spawn was located, it was observed and video recorded for about 10–15 minutes to report spawn visitors. The location of the foam nest and the height from the ground level to the nest were recorded. The spawns were examined daily at both selected localities until the embryos developed into tadpoles. A plastic container filled with 1,000ml of dechlorinated water was kept below each egg mass to collect emerging tadpoles. Observations were made at 24-hour intervals and the developed tadpoles were released to the respective water sources after recording the number. A similar procedure was followed for both infected and non-infected spawns. The presence of maggots, color changes, and the shape of the foam nests were used to distinguish infected nests from uninfected ones. Three severely infected spawns were carefully removed from the attached substrates and brought to the Insectary, Department of Zoology, University of Peradeniya for further investigations. At the laboratory, the foam nests were placed in dechlorinated water in a tray and transferred to fine-mesh mosquito rearing cages (50 × 50 × 50 cm) for maintenance of the fly colonies (at 25°C temperature, 75% relative humidity, and 12 D: 12 L photoperiodicity). Emerged flies were euthanized at -20°C in a freezer and pinned for identification. Morphological identification was done using the standard taxonomic key in Rognes (2015).

To confirm the identity of the dipteran species, DNA barcoding was also performed. DNA was extracted from some of the collected flies following Livak (1984). The mitochondrial Cytochrome Oxidase I (COI) gene was amplified using the previously described primers C1–J–1718F (5'–GGA GGA TTT GGA AAT TGA TTA GTT CC–3') and C1–N–2191R (5'–CCC GGT AAA ATT AAA ATA TAA ACT TC–3') (Simon et al. 1994). PCR amplification was done in a thermal cycler (Techne–Flexigene, England) following Nolan et al. (2007). Positive PCR products were sequenced using an automatic DNA sequencer (Applied Biosystems Series 3500, U.S.A.) in the Department of Molecular Biology and Biotechnology, University of Peradeniya. The sequence trace files were manually inspected using MEGA V7 (Kumar et al. 2016) and low-quality sequences were excluded from the analysis. The DNA sequences were annotated using the GenBank database (https://www.ncbi.nlm.nih.gov/) and BLASTn tool. The newly generated sequences were deposited in GenBank under the accession numbers MN786865 and MN786866.

The dissection and examination of male genitalia were done following Rognes (2009). The tip of the abdomen (from tergite 4) was removed and transferred to a 10% potassium hydroxide solution, then heated in a water bath for about 20 minutes. The abdomen was then transferred to distilled water and rinsed with 95% ethanol for 10 minutes to fix the integument. The male genital organs were separated using fine forceps, for preparation of microscopic slides. The separated male genitalia were mounted using Canada Balsam, and photographs of the prepared slides were taken using an Olympus BX53 Digital Upright Microscope (Olympus Corporation, Florida, USA).

Morphological identification confirmed that the emerged flies belonged to *Caiusa testacea* Senior-White, 1923 of the family Calliphoridae. According to Rognes (2015), the following morphological features were

Image 1. Infected and non-infected foam nests of *Polypedates cruciger* and different life stages of *Caiusa testacea* flies: a—adult *Polypedates cruciger* | b—uninfected fresh foam nest attached to a *Polyscias scutellaria* leaf | c—tadpoles from an uninfected foam nest | d—putrefying foam nest due to *C. testacea* infection | e—*C. testacea* fly on a fresh foam nest | f—*C. testacea* 3rd instar larva | g—*C. testacea* pupae | h—lateral aspect of adult *C. testacea* fly. © a,b—Anslem de Silva; c-h— W.G.D. Chathuranga.

identified for them. Cerci short, backwardly bent, and with a pronounced distal separation between the apices in dorsal view. Base of cerci wide proximal to separation (Image 2A). In lateral view, surstylus rather broad and short, very gently curved below. Thoracic dorsum yellow and tergites 4 and 5 of abdomen with slight darkening and lack of metallic bluish sheen (Image 2D). A BLAST search of the GenBank database showed a 96.92% identity to available *Caiusa testacea* sequences together with a 100% query cover.

A total of 24 spawns of *P. cruciger* were studied (Image 1a–1d). Observations were carried out on 10 spawns in Gampola (including the three collected spawns) and 14 spawns from the Peradeniya study site. These spawns were located at a height of 0.1–3.0 m above the ground. Plant species such as *Polyscias scutellaria* (Araliaceae), *Nelumbo nucifera* (Nelumbonaceae), *Gliricidia sepium* (Fabaceae), *Echinodorus palifolius* (Alismataceae), *Persea americana* (Lauraceae), and artificial substrates including cement walls, metal wire mesh, ceiling sheets, metal or plastic pipes just above a water source, were the most common spawning sites of *P. cruciger*. Of the examined spawns, 16 (66.7%) were not infected while eight (33.3%) were infected with fly larvae (Image 1f). All the infected spawns were reported from the Gampola study location, representing 80% of the total.

During this study, we observed oviposition of *C. testacea* flies only three times (Image 1e) on fresh foam nests of *P. cruciger*, and the larvae of *C. testacea* emerged from two-day-old infected spawns. An average of 354 ± 67 embryos developed into tadpoles (Image 1c) from healthy spawns (n=15), except one that produced an exceptionally high number of tadpoles (approximately 800). When compared with the healthy spawns, none of the embryos of the infected spawns (n=8) developed into tadpoles (Image 1d). According to our observation of eight infected spawns, approximately 400 embryos were destroyed with a single nest infestation. An average of 52 ± 9 *C. testacea* larvae pupariated (Image 1g) and 17 ± 8 emerged as adults from the three collected spawns (Image 1h). Accordingly, an average of 33% (17/52) of the larvae were able to complete their life cycle from a single spawn. The 1st to 3rd instar larval stages of the fly lasted around 6–7 days, while the puparial period lasted 8–11 days. The life cycle of *C. testacea* was completed within 18 to 20 days. Emerged adult flies were freeze-killed and pinned for identification. Larval instars, puparia, and a few adults of *C. testacea* were also preserved in 70% ethanol as voucher specimens and deposited in the Zoonotic and Disease Ecology Laboratory of the Department of Zoology, University of Peradeniya, Sri Lanka. Different morphological body aspects of *C. testacea*, including taxonomic features, are shown in Images 1h, 2A–2D.

Our study highlights the threat caused by *C. testacea* flies to the foam nests of *Polypedates cruciger* frogs and provides an indication of the major impact of these flies on the population dynamics of *P. cruciger*. Even though studies have reported the impact of predatory pressure causing the population decline of amphibians (Lin & Lue 2000; Kiesecker 2003), it has not been listed as a priority factor in conservation approaches in Sri

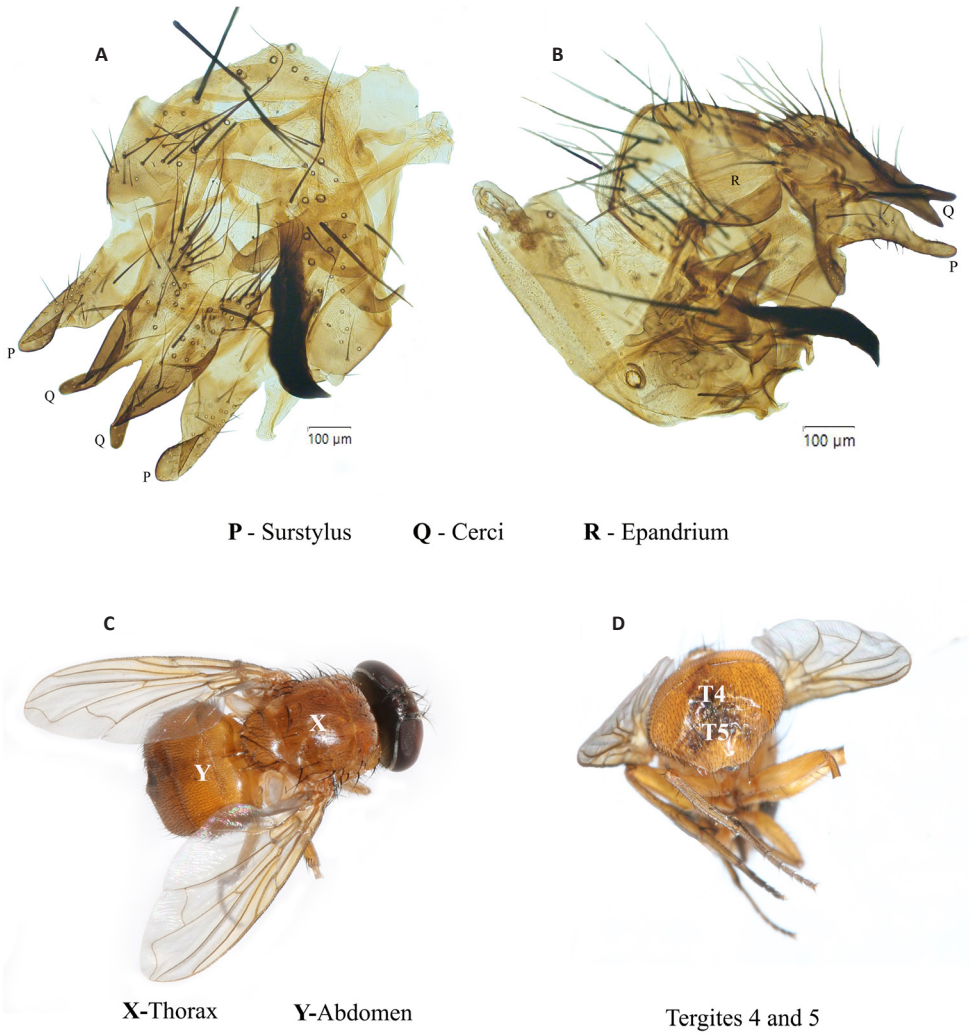

Image 2. Morphological features of *Caiusa testacea* flies: A—posterior aspect of the genitalia |B—lateral aspect of the genitalia | C—yellowish thorax and abdomen | D—yellowish T4 and T5 with slightly darkened patch. © a,b—W.G.D. Chathuranga; c-d—Kumudu Wijesooriya.

Lanka. In this study, we provide data on the natural predatory pressure of the calliphorid fly *Caiusa testacea* on the population structure of the rhacophorid tree frog *Polypedates cruciger*. Further, our results provide evidence of natural threats of Rhacophoridae anurans in Sri Lanka.

The presence of these flies had been reported from Sri Lanka, India, and Nepal by Rognes (2015), however, these flies had not been identified as egg predators of Sri Lankan Rhacophoridae species by any of the earlier studies. Our results reveal that larvae of *C. testacea* flies destructively consume eggs and embryos of *P. cruciger*. In an earlier study, *Caiusa indica* was identified as an egg predator of *P. cruciger* in Sri Lanka (Morgan-Davies 1958); however, previous studies had not identified *C. testacea* as a predator of foam nests of Rhacophoridae, and this is the first study that reports on the feeding behavior and the life history of *C. testacea*.

Rognes (2015) estimated that the time from the infestation of spawns by *Caiusa* flies to the completion of metamorphosis is nearly a week. In contrast, we observed a relatively longer developmental period, where *C. testacea* flies complete metamorphosis within three weeks. Lin et al. (2000) and Lin & Lue (2000) described the oviposition behavior of *Caiusa violacea* (as *C. coomani*). According to those authors, the flies lay their eggs when the outer surface of the foam nest is soft, within a few hours after the foam nest is formed. Similarly, Banerjee et al. (2018) reported that *Caiusa* flies lay their eggs on foam nests seven hours after the construction of the nest. Our study confirmed the oviposition of *C. testacea* flies on fresh foam nests of *P. cruciger* (Image 1e), however, we were not able to provide more specific information about the timeframe

during which the flies are attracted to the nests. Our observations showed that larvae appeared within 2 to 3 days after oviposition and that the life cycle was completed (to metamorphosis) within 18 days.

Rognes (2015) reported that most of the dipteran predators of foam nests are able to respond to chemical cues released from the fresh foam nests built by the frogs. Thus, the gravid females of *C. testacea* flies may respond to chemical cues of freshly formed foam nests or chemical signals produced by *P. cruciger* frogs during spawning. Our data could not, however, confirm this hypothesis. There are interesting hypotheses explaining the selection of foam nests by dipteran flies as oviposition sites. For example, Banerjee et al. (2018) hypothesized that the frog eggs represent easier prey for *Caiusa* larvae compared to mobile tadpoles, which may allow these flies to overcome environmental constraints and resource limitations.

The distribution of *P. cruciger* extends 1,500m in the wet zone of central and southwestern parts of Sri Lanka (De Silva & De Silva 2000). *Caiusa testacea* has also been reported from similar locations in the central part of Sri Lanka, including Maskeliya, Suduganga, Kandy, and Niroddumunai (Rognes 2015), where *P. cruciger* is also reported. This habitat overlap of the predatory flies and *P. cruciger* may have driven the evolution of the predatory behavior of this fly species on the foam nests of *P. cruciger*. At the same time, this habitat overlap may negatively affect *P. cruciger* as it gives more opportunities for *C. testacea* flies to attack their nests. According to IUCN Red list 2012 categories, *P. cruciger* is listed as a Least Concern (LC) anuran species (Manamendra-Arachchi & Meegaskumbura 2012); however, the continual increase of anthropogenic impacts and changing climatic factors, together with infestations of *C. testacea*, may negatively affect *P. cruciger* populations, causing it to become a 'threatened species'. Furthermore, Sri Lanka harbors four more foam nesting anuran species in the family Rhacophoridae [(*Polypedates maculates* Gray 1830, *Taruga eques* Günther, 1858, *Taruga fastigo* (Manamendra-Arachchi & Pethiyagoda, 2001), and *Taruga longinasus* (Ahl, 1927)] (Meegaskumbura et al. 2010). As a result, there are possibilities for all other foam nesting Rhacophoridae anurans to be endangered by nest predation by *Caiusa testacea* flies. As we have seen the habitat overlap of Rhacophoridae species and these flies, there is a high chance of egg predation by *Caiusa* on these tree frogs in Sri Lanka. A proper understanding of the biology, distribution, and population assessments of both *C. testacea* and *P. cruciger*, however, will be vital in assessing the threats of *C. testacea* flies on the population dynamics of *P. cruciger* in the country.

In summary, we report *C. testacea* as a predator of foam nests of *P. cruciger* frogs of the family Rhacophoridae in Sri Lanka for the first time. More importantly, we recognize the predatory pressure of these flies on spawns of *P. cruciger*, highlighting their needful consideration in conservation approaches concerning these frogs.

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